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PATENT APPLICATION

HUMAN *RRN3* AND COMPOSITIONS AND METHODS RELATING THERETO

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CROSS-REFERENCES TO RELATED APPLICATIONS

This application claims priority from Provisional Application U.S. Serial No. 60/225,893, filed August 16, 2000, the disclosure of which is incorporated herein by reference.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

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BACKGROUND OF THE INVENTION

The ribosomal RNA genes ("rRNA") of eukaryotic cells are transcribed by an enzyme solely dedicated to that purpose, RNA polymerase I ("pol I"). The expression of the rRNA genes is coordinated with cellular proliferation. When cell growth is impaired by nutrient deprivation or depletion, transcription of the rRNA genes declines. This decline is reversed when growth-permissive conditions are restored. Since growth-rate dependence is a universal feature of rRNA gene regulation, identifying the molecular mechanisms that couple pol I activity to cell growth is a central question in studies of all eukaryotic systems, including the yeast and mammalian systems.

Transcription of rRNA genes of the yeast *Saccharomyces cerevisiae* requires the activity of at least three transcription factors, which have been defined both genetically and biochemically. Two of these factors, Core Factor and Upstream Activation Factor, are multi-subunit factors that interact directly with distinct elements of the rRNA promoter to assemble a preinitiation complex. Core Factor ("CF") is composed of three essential gene products, Rrn6, Rrn7, and Rrn11, and associates with TATA box binding protein ("TBP"). CF is required to direct transcription initiation from the core promoter of an rRNA gene both *in vitro* and *in vivo* (*see* Keys *et al.*, *Genes Dev.* 8:2349-62 (1994); Lin *et al.*, *Mol. Cell. Biol.* 16:6436-43 (1996); Steffan *et al.*, *Genes Dev.*

10:2551-63 (1996); Keener *et al.*, *J. Biol. Chem.* 273:33795-802 (1998); Lalo *et al.*, *J. Biol. Chem. 271*:21062-67 (1996)). Upstream Activation Factor ("UAF") binds to the upstream promoter element and stimulates transcription from the core promoter. When the yeast genes encoding the UAF subunits Rrn5, Rrn9, or Rrn10 are individually disrupted, cells remain viable but exhibit pronounced growth defects, indicating that UAF activity is necessary to support levels of rRNA synthesis required for normal cell growth (*see* Keys *et al.*, *Genes Dev.* 10:887-903 (1996)). UAF subunits interact with CF subunits *in vitro*, and direct interaction of UAF with TBP has been shown to mediate transcriptional activation *in vivo* (*see* Steffan *et al.* (1996), *supra*; Steffan *et al.*, *Mol. Cell. Biol.* 18:3752-61 (1998)).

A third transcription factor, Rrn3, is unique in that it functions as a single subunit, shows no sequence-specific DNA binding activity, and is not required for pre-initiation complex assembly (*see* Yamamoto *et al.*, *EMBO J.* 15:3964-73 (1996)). The Rrn3 protein appears instead to function by direct interaction with RNA polymerase I since it is stably associated with pol I in transcriptionally active extracts (*see* Milkereit and Tschochner, *EMBO J.* 17:3692-703 (1998)). The transcriptional activity of pol I is enhanced by pre-incubation with Rrn3 protein in the absence of either DNA template or other pol I transcription factors (*see* Yamamoto *et al.* (1996), *supra*; Keener *et al.* (1998), *supra*). The interaction of Rrn3 polypeptide with RNA polymerase I fluctuates with changes in cellular growth rate; Rrn3 polypeptide is not associated with pol I in transcriptionally-inactive extracts prepared from growth-arrested cells. Transcriptional activity is restored upon addition of Rrn3-associated pol I purified from growing cells (*see* Milkereit and Tschochner, *supra*). These observations suggest that Rrn3 activity may be regulated in a growth-dependent manner. Although the specific function of Rrn3 polypeptide is as yet unknown, it is essential for rRNA gene transcription *in vivo* and *in vitro*, and it may be required to mediate productive interactions of pol I with the pre-initiation complex.

Transcription of mammalian rRNA genes also requires two promoter-binding transcription factors which appear to perform functions similar to those of the yeast pol I factors. The mammalian transcription factors are not conserved with those of yeast. The core promoter-binding factor TIF-IB/SL1, which is essential for transcription, is comprised of TBP and three transcription-associated factors ("TAF's"). The TAF's are

similar in size to the three yeast CF subunits, but display no amino acid sequence similarity to their yeast counterparts (*see* Comai *et al.*, *Cell* 68:965-76 (1992); Eberhard *et al.*, *Nucleic Acids Res.* 21:4180-86 (1993); Heix *et al.*, *Proc. Natl. Acad. Sci. USA* 94:1733-38 (1997)). The upstream stimulatory activity in mammalian cells is mediated by a single protein, UBF, which bears no resemblance to the multi-subunit yeast UAF complex (*see* Bell *et al.*, *Science* 241:1192-97 (1988); Schnapp and Grummt, *J. Biol. Chem.* 266:24588-95 (1991)). DNA binding by UBF is mediated by high mobility group domains which are not present in any of the yeast pol I transcription factors, and no proteins related to the yeast UAF subunits have been identified in mammals to date. It therefore appears that the promoter-binding factors of yeast and mammals are evolutionarily divergent.

In mammalian cells, two RNA polymerase I-associated factors, TIF-IA and TIF-IC, have been identified (*see* Schnapp *et al.*, *EMBO J.* 9:2857-63 (1990); Schnapp *et al.*, *EMBO J.* 13:4028-35 (1994)). Like yeast Rrn3 ("yRrn3"), TIF-IA and TIF-IC are not required for pre-initiation complex assembly but are essential for transcription initiation by pol I (*see* Schnapp and Grummt, *supra*). The relationship of these factors to yRrn3 has not yet been determined since their genes have not yet been isolated. However, TIF-IA shares an important functional similarity with yRrn3 in that its activity is regulated by cellular growth rate (*see* Schnapp *et al.* (1990), *supra*; Schnapp *et al.*, *Mol. Cell. Biol.* 13:6723-32 (1993)).

rRNA synthesis is required for cell division and differentiation, and also for normal cellular metabolism. The assembly of new ribosomal subunits requires new rRNA transcripts. Thus, rRNA transcription provides a common regulatory point for controlling cell proliferation in a wide variety of cell types. Reagents that modulate rRNA transcription could be used to affect proliferation in such a wide variety of cell types, and would not be limited to current reagents that are cell-type specific. For example, reagents, and methods of their use, that modulate rRNA synthesis could be used to stimulate hypoproliferative cells or to inhibit hyperproliferative cells, in diseases such as cancer. There is need, therefore, for reagents, and methods of using such reagents, to modulate cell proliferation through rRNA transcription. Surprisingly, the present invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

The present invention relates to the discovery, identification and characterization of eukaryotic *RRN3* genes. The invention encompasses nucleotide sequences of the *RRN3* gene and amino acid sequences of its encoded polypeptide product, as well as fragments, derivatives and analogs thereof. The invention also encompasses the production of Rrn3 polypeptides and antigen-specific antibodies. The invention further encompasses compositions and methods for screening, diagnostic and therapeutic applications.

One aspect relates to *RRN3* nucleic acids, including mRNAs, DNAs, cDNAs, genomic DNA, as well as *RRN3* antisense nucleic acids. Such nucleic acids include the *RRN3* cDNA having the nucleotide sequence of SEQ ID NO:1. Another aspect relates to *RRN3* nucleic acid derivatives or fragments that encode Rrn3 polypeptides, or portions thereof. Such derivatives include nucleic acids encoding all possible codon choices for the same amino acid or conservative amino acid substitutions thereof. Other *RRN3* nucleic acids include those nucleic acids that are capable of selectively hybridizing to a human *RRN3* cDNA (*e.g.*, SEQ ID NO:1) under stringent hybridization conditions. A related aspect of the present invention relates to nucleic acid probes comprising polynucleotides of sufficient length to selectively hybridize to a polynucleotide encoding an Rrn3 polypeptide of the present invention.

In another aspect, the present invention provides substantially pure preparations of human Rrn3 and polypeptide fragments, derivatives and analogs thereof. In a related aspect, the invention concerns nucleic acid constructs for expressing the *RRN3* nucleic acids. Such expression constructs typically comprise a transcriptional promoter, a nucleic acid which encodes the Rrn3 polypeptide, derivative or fragment thereof, and a transcriptional terminator, each operably linked for expression of the Rrn3 polypeptide, derivative or fragment. In another aspect, the invention provides the ability to produce Rrn3 polypeptides, derivatives, or fragments thereof by recombinant means, typically in cultured eukaryotic cells or in prokaryotic cells. The expressed Rrn3 polypeptide, derivatives or fragments can have the same functional activity as the corresponding native Rrn3 polypeptide, or an altered activity. Accordingly, isolated and purified polynucleotides are described which encode Rrn3 polypeptide, derivatives, and fragments

thereof, where the polynucleotides can be in the form of *RRN3* nucleic acids, such as genomic DNA, cDNA, or mRNA.

In another aspect, the *RRN3* nucleic acids can be used to identify other mammalian genes that encode Rrn3-like molecules. The *RRN3* nucleic acids can also be used to screen for mutations in a *RRN3* gene that are associated with certain diseases. As such, the invention further relates to materials and methods for the identification of disease-associated mutations, where the *RRN3* nucleic acids are used to detect the presence of mutations in a biological sample. *RRN3* nucleic acid probes can also be used to identify mutations in a *RRN3* gene for diagnostic purposes. The probes can be full-length genomic DNA, cDNA, RNA or nucleic acids as small as from about 14 to 25 nucleotides, more often though from about 40 to about 50, or more nucleotides in length.

The invention also provides antibodies to Rrn3 polypeptide, in the form of polyclonal or monoclonal antibodies. Such antibodies can specifically bind to an Rrn3 polypeptide or fragment, derivative or analog thereof, and can be incubated with a biological sample under conditions conducive to immune complex formation, such as by ELISA. The resulting complexes can then be detected, typically by means of a label such as an enzyme, fluorophore, radionuclide, chemiluminescent, particle, or a second labeled antibody. Thus, means are provided for immunohistochemical staining of tissues, including tumor biopsies. The invention further relates to methods of identifying agonists and antagonists that modulate the activity of an Rrn3 polypeptide, and further provides a method for identifying agents that specifically affect one Rrn3 polypeptide, without affecting other Rrn3 polypeptides.

A further understanding of the nature and advantages on the invention will become apparent by reference to the remaining portions of the specification.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Prior to setting forth the invention in more detail, it may be helpful to a further understanding thereof to set forth definitions of certain terms as used hereinafter.

Definitions:

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to

which this invention belongs. Although any methods and materials similar to those described herein can be used in the practice or testing of the present invention, only exemplary methods and materials are described. For purposes of the present invention, the following terms are defined below.

The term "*RRN3* locus" and "*RRN3* gene" refer to the coding sequences, intervening sequences and regulatory elements controlling transcription and/or translation. The terms "*RRN3* locus" and "*RRN3* gene" include all allelic variations of *RRN3*. A wild-type *RRN3* sequence refers to the sequence of SEQ ID NO:1.

The term "*RRN3* nucleic acids" refers to polynucleotides from the *RRN3* locus, such as those encoding Rrn3 polypeptides, including mRNAs, DNAs, cDNAs, genomic DNA, as well as antisense nucleic acids, and polynucleotides encoding fragments, derivatives and analogs thereof. Useful fragments and derivatives include those based on all possible codon choices for the same amino acid, and codon choices based on conservative amino acid substitutions. Useful derivatives further include those having at least 50% or at least 70% polynucleotide sequence identity, and typically 80%, more typically 90% sequence identity, to the *RRN3* nucleic acid of SEQ ID NO:1.

The terms "polynucleotide" and "nucleic acid" refer to a polymer composed of a multiplicity of nucleotide units (ribonucleotide or deoxyribonucleotide or related structural variants) linked via phosphodiester bonds. A polynucleotide or nucleic acid can be of substantially any length, typically from about six (6) nucleotides to about 10^9 nucleotides or larger. Polynucleotides and nucleic acids include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and can also be chemically or biochemically modified or can contain non-natural or derivatized nucleotide bases, as will be readily appreciated by the skilled artisan. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, and the like), charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, and the like), pendent moieties (*e.g.*, polypeptides), intercalators (*e.g.*, acridine, psoralen, and the like), chelators, alkylators, and modified linkages (*e.g.*, alpha anomeric nucleic acids, and the like). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical

interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

The term "oligonucleotide" refers to a polynucleotide of from about six (6) to about one hundred (100) nucleotides or more in length. Thus, oligonucleotides are a subset of polynucleotides. Oligonucleotides can be synthesized on an automated oligonucleotide synthesizer (for example, those manufactured by Applied BioSystems (Foster City, CA)) according to specifications provided by the manufacturer.

The term "primer" as used herein refers to a polynucleotide, typically an oligonucleotide, whether occurring naturally, as in an enzyme digest, or whether produced synthetically, which acts as a point of initiation of polynucleotide synthesis when used under conditions in which a primer extension product is synthesized. A primer can be single-stranded or double-stranded.

"Rrn3 polypeptide" refers to a polypeptide encoded by the *RRN3* locus, and fragments, derivatives or analogs thereof. The term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins are included within the definition of a polypeptide. A "fragment" refers to a portion of a polypeptide typically having at least 10 contiguous amino acids, more typically at least 20, still more typically at least 50 contiguous amino acids of the Rrn3 polypeptide. A derivative is a polypeptide having conservative amino acid substitutions, as compared with another sequence. Derivatives further include, for example, glycosylations, acetylations, phosphorylations, and the like. Further included within the definition of "polypeptide" are, for example, polypeptides containing one or more analogs of an amino acid (*e.g.*, unnatural amino acids, and the like), polypeptides with substituted linkages as well as other modifications known in the art, both naturally and non-naturally occurring. Ordinarily, such polypeptides will be at least about 50% identical to the native Rrn3 amino acid sequence, typically in excess of about 90%, and more typically at least about 95% identical.

The terms "amino acid" or "amino acid residue", as used herein, refer to naturally occurring L amino acids or to D amino acids as described further below. The commonly used one- and three-letter abbreviations for amino acids are used herein (*see, e.g.*, Alberts *et al.*, *Molecular Biology of the Cell*, 3d ed., Garland Publishing, Inc., New York (1994)).

The term "heterologous" refers to a nucleic acid or polypeptide from a different source, (e.g. a tissue, organism or species), as compared with another nucleic acid or polypeptide.

The term "isolated" refers to a nucleic acid or polypeptide that has been removed from its natural cellular environment. An isolated nucleic acid is typically at least partially purified from other cellular nucleic acids, polypeptides and other constituents.

The term "functionally active" Rrn3 polypeptides refers to those fragments, derivatives and analogs displaying one or more known functional activities associated with a full-length (wild-type) Rrn3 polypeptide (*e.g.*, stimulating rRNA transcription (*i.e.* synthesis), binding to RNA polymerase I, or other Rrn3 binding partner), antigenicity (binding to an anti-Rrn3 antibody), immunogenicity, and the like. Functionally active molecules include Rrn3 polypeptides, fragments, derivatives and analogs thereof; nucleic acids encoding Rrn3 polypeptides, fragments, and derivatives thereof; and anti-Rrn3 antibodies.

The term "therapeutically effective" refers to an amount of a molecule (*e.g.*, an Rrn3 polypeptide, anti-Rrn3 antibody, or *RRN3* nucleic acid) that is sufficient to modulate rRNA transcription in a subject, such as a patient or a mammal.

The terms "identical" or "percent identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acid residues that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms, or by visual inspection.

The phrase "substantially identical," in the context of two nucleic acids or polypeptides, refers to two or more sequences or subsequences that have at least 60%, typically 80%, most typically 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms, or by visual inspection. An indication that two polypeptide sequences are "substantially identical" is that one polypeptide is immunologically reactive with antibodies raised against the second polypeptide.

"Similarity" or "percent similarity" in the context of two or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a

specified percentage of amino acid residues or conservative substitutions thereof, that are the same, when compared and aligned for maximum correspondence, as measured using one of the following sequence comparison algorithms, or by visual inspection. By way of example, a first amino acid sequence can be considered similar to a second amino acid sequence when the first amino acid sequence is at least 50%, 60%, 70%, 75%, 80%, 90%, or even 95% identical, or conservatively substituted, to the second amino acid sequence when compared to an equal number of amino acids as the number contained in the first sequence, or when compared to an alignment of polypeptides that has been aligned by a computer similarity program known in the art, as discussed below.

The term "substantial similarity" in the context of polypeptide sequences, indicates that the polypeptide comprises a sequence with at least 70% sequence identity to a reference sequence, or preferably 80%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. In the context of amino acid sequences, "substantial similarity" further includes conservative substitutions of amino acids. Thus, a polypeptide is substantially similar to a second polypeptide, for example, where the two peptides differ by one or more conservative substitutions.

The term "conservative substitution," when describing a polypeptide, refers to a change in the amino acid composition of the polypeptide that does not substantially alter the polypeptide's activity. Thus, a "conservative substitution" of a particular amino acid sequence refers to substitution of those amino acids that are not critical for polypeptide activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, etc.) such that the substitution of even critical amino acids does not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, the following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). (*See also* Creighton, *Proteins*, W. H. Freeman and Company (1984).) In addition, individual substitutions, deletions or

additions that alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservative substitutions."

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (*Adv. Appl. Math.* 2:482 (1981), which is incorporated by reference herein), by the homology alignment algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-53 (1970), which is incorporated by reference herein), by the search for similarity method of Pearson and Lipman (*Proc. Natl. Acad. Sci. USA* 85:2444-48 (1988), which is incorporated by reference herein), by computerized implementations of these algorithms (*e.g.*, GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection. (*See generally* Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, 4th ed., John Wiley and Sons, New York (1999)).

One example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments to show the percent sequence identity. It also plots a tree or dendogram showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng and Doolittle (*J. Mol. Evol.* 25:351-60 (1987), which is incorporated by reference herein). The method used is similar to the method described by Higgins and Sharp (*Comput. Appl. Biosci.* 5:151-53 (1989), which is incorporated by reference herein). The program can align up to 300 sequences, each of a maximum length of 5,000 nucleotides or amino acids. The multiple alignment procedure begins with the pairwise alignment of the two most similar sequences, producing a cluster of two aligned sequences. This cluster is then aligned to the next most related sequence or cluster of aligned sequences. Two clusters of sequences are aligned by

a simple extension of the pairwise alignment of two individual sequences. The final alignment is achieved by a series of progressive, pairwise alignments. The program is run by designating specific sequences and their amino acid or nucleotide coordinates for regions of sequence comparison and by designating the program parameters. For example, a reference sequence can be compared to other test sequences to determine the percent sequence identity relationship using the following parameters: default gap weight (3.00), default gap length weight (0.10), and weighted end gaps.

Another example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described by Altschul *et al.* (*J. Mol. Biol.* 215:403-410 (1990), which is incorporated by reference herein). (*See also* Zhang *et al.*, *Nucleic Acid Res.* 26:3986-90 (1998); Altschul *et al.*, *Nucleic Acid Res.* 25:3389-402 (1997), which are incorporated by reference herein). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.* (1990), *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction is halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-9 (1992), which is incorporated by reference herein) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-77 (1993), which is incorporated

by reference herein). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more typically less than about 0.01, and most typically less than about 0.001.

A further indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions.

The term "immunological cross-reactive" means that a polypeptide, fragment, derivative or analog is capable of competitively inhibiting the binding of an antibody to its antigen.

The terms "transformation" or "transfection" refer to the process of stably altering the genotype of a recipient cell or microorganism by the introduction of polynucleotides. This is typically detected by a change in the phenotype of the recipient cell or organism. The term "transformation" is generally applied to microorganisms, while "transfection" is used to describe this process in cells derived from multicellular organisms.

The term "sample" generally indicates a specimen of tissue, cells, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, blood cells, hair, tumors, organs, other material of biological origin that contains polynucleotides, or *in vitro* cell culture constituents of any of these. A sample can further be semi-purified or purified forms of polynucleotides. A sample can be isolated from a mammal, such as a human, an animal, or any other organism having a *RRN3* locus, as well as *in vitro* culture constituents of any of these.

The term "proliferation" refers to activities such as transformation, a change in gene expression, and other changes in cell state that are dependent, directly or indirectly, on new ribosomal RNA synthesis, new ribosome assembly, or new protein synthesis requiring new ribosome assembly. "Hyperproliferation" refers to an increase in

one or more proliferative activities, as compared with normal tissue. "Hypoproliferation" refers to an decrease in one or more proliferative activities, as compared with normal tissue.

The term "disease" refers to a disease, condition, or disorder associated with hyperproliferation or hypoproliferation. Diseases involving hyperproliferation include, but are not limited to, cancer, malignancies, premalignant conditions (*e.g.*, hyperplasia, metaplasia, dysplasia), benign tumors, hyperproliferative disorders, benign dysproliferative disorders, autoimmune diseases, and the like. Diseases involving cell hypoproliferation include, but are not limited to, cardiac disease and other conditions in which an increase in cell proliferation is desired.

Generally, other nomenclature used herein and many of the laboratory procedures in cell culture, molecular genetics and nucleic acid chemistry and hybridization, which are described below, are those well known and commonly employed in the art. (*See generally* Ausubel *et al.* (1999) *supra*; Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 3d Ed., Cold Spring Harbor Laboratory Press, New York (2001), which are incorporated by reference herein). Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, preparation of biological samples, preparation of cDNA fragments, isolation of mRNA and the like. Generally enzymatic reactions and purification steps are performed according to the manufacturers' specifications.

Genetic methods suitable for manipulating yeast strains are generally described in Sherman *et al.*, *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor, N.Y. (1983)) and "Guide to Yeast Genetics and Molecular Biology" In *Methods in Enzymology* (Guthrie and Fink (eds.), Academic Press, San Diego, CA (1991), which are incorporated by reference herein).

The *RRN3* Gene:

The invention relates to the nucleotide sequences of human *RRN3*. The human *RRN3* cDNA (SEQ ID NO:1) was isolated by a BLAST search using the yeast Rrn3 amino acid sequence as a reference to identify expressed sequence tags ("EST's") corresponding to the human *RRN3* gene. The *RRN3* cDNA sequence was assembled from the EST's identified by database searching. The *RRN3* cDNA encodes a polypeptide of

651 amino acids (SEQ ID NO:2). Analysis of the corresponding polypeptide revealed that it has an apparent molecular weight of about 74 kD, as determined by SDS PAGE, which is consistent with the predicted molecular weight. The human Rrn3 polypeptide is 21% identical and 43% similar to yeast Rrn3 polypeptide. Amino acid conservation is distributed throughout the length of the two polypeptides. Database searching reveals that the human *RRN3* cDNA is located on chromosome 16 in a region of greater than 26 kb in length and which contains at least fifteen introns. Expressed sequence tags arising from the *RRN3* gene have been isolated from a variety of tissues, including lung, retina, thymus, and prostate. Ubiquitous expression of Rrn3 polypeptide is consistent with its role as a pol I transcription factor for rRNA synthesis.

Three regions of Rrn3 polypeptides are shared by all Rrn3 family members, but are not found in other proteins. These regions are conserved in both sequence and length and display the general consensus sequences:

(1) Tyr(Ile/Leu)(Ala/Gly)(Ala/Ser)(Phe/Tyr)(Ile/Leu)(Ala/Ser)ArgAlaLys;

(2) PheTyr(Ala/Ser)XaaXaaGln(Ala/Ser)(Ile/Leu)XaaXaaXaa (Phe)XaaPheArg; and

(3) PhePro(Phe/Tyr)AspXaaXaaXaaLeu(Lys);

where parentheses indicate positions which vary among family members, and Xaa indicates variable positions. As these motifs are not found in the sequences of other polypeptides, their evolutionary conservation reflects the pol I-specific function of Rrn3 polypeptides. Since yeast Rrn3 polypeptide does not bind to DNA, these regions are likely to mediate protein-protein interactions with other components of the pol I transcriptional machinery.

In a specific embodiment, *RRN3* nucleic acids comprise the cDNA sequence of SEQ ID NO:1, or the coding region of the *RRN3* locus, or nucleic acid sequences (*e.g.*, an open reading frame) encoding a Rrn3 polypeptide (SEQ ID NO:2). *RRN3* nucleic acids further include mRNAs, genomic DNA, and antisense nucleic acids corresponding to the *RRN3* locus. *RRN3* nucleic acids further include derivatives (*e.g.*, nucleotide sequence variants), such as those encoding other possible codon choices for the same amino acid or conservative amino acid substitutions thereof, such as naturally occurring allelic variants. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *RRN3*

gene, for example, SEQ ID NO:1, can be used in the practice of the present invention. These include, but are not limited to, nucleotide sequences comprising all or portions of a *RRN3* gene which is altered by the substitution of different codons that encode the same or a functionally equivalent amino acid residue (*e.g.*, a conservative substitution) within the sequence, thus producing a silent change.

The invention also provides *RRN3* nucleic acid fragments of at least 6 contiguous nucleotides (*e.g.*, a hybridizable portion); in other embodiments, the nucleic acids comprise at least 8 contiguous nucleotides, 25 nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, 200 nucleotides, or even up to 250 nucleotides or more of a *RRN3* sequence. In another embodiment, the nucleic acids are smaller than 200 or 250 nucleotides in length. The *RRN3* nucleic acids can be single or double-stranded. As is readily apparent, as used herein, a "nucleic acid encoding a fragment of an Rrn3 polypeptide" is construed as referring to a nucleic acid encoding only the recited fragment or portion of the Rrn3 polypeptide and not the other contiguous portions of the Rrn3 polypeptide as a contiguous sequence. Fragments of *RRN3* nucleic acids encoding one or more Rrn3 domains are also provided.

RRN3 nucleic acids further include those nucleic acids hybridizable to, or complementary to, the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, 200, or 250 nucleotides or more of a *RRN3* gene. In a specific embodiment, a nucleic acid which is hybridizable to a *RRN3* nucleic acid (*e.g.*, having sequence SEQ ID NO:1), or to a nucleic acid encoding a *RRN3* derivative, under conditions of low, medium or high stringency, is provided.

By way of example, and not limitation, procedures using low stringency conditions are as follows: Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5x SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% polyvinylpyrrolidone (PVP), 0.1% Ficoll, 1% bovine serum albumin (BSA), and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 x 10^6 cpm ^{32}P-labeled probe. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2x SSC, 25 mM Tris-HCl (pH 7.4), 5 mM

EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency that can be used are well known in the art (*e.g.*, those employed for cross-species hybridizations). (*See also* Shilo and Weinberg, *Proc. Natl. Acad. Sci. USA* 78:6789-92 (1981)).

In another embodiment, a nucleic acid which is hybridizable to a *RRN3* nucleic acid under conditions of high stringency is provided. By way of example, and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6x SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 65°C for 1 hour in a solution containing 2x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1x SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which can be used are well known in the art. (*See generally* Ausubel *et al.*, *supra;* Sambrook *et al.*, *supra*).

In another specific embodiment, a nucleic acid which is hybridizable to a *RRN3* nucleic acid under conditions of moderate stringency is provided. By way of example, and not limitation, procedures using such conditions of moderate stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 55°C in buffer composed of 6x SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.2% Ficoll, 0.02% BSA and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 24 hours at 55°C in a prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA.

Various other stringency conditions which promote hybridization can be used. For example, hybridization in 6x SSC at about 45°C, followed by washing in 2x SSC at 50°C can be used. Alternatively, the salt concentration in the wash step can range from low stringency of about 5x SSC at 50°C, to moderate stringency of about 2x SSC at

50°C, to high stringency of about 0.2x SSC at 50°C. In addition, the temperature of the wash step can be increased from low stringency conditions at room temperature, to moderately stringent conditions at about 42°C, to high stringency conditions at about 65°C. Other conditions include, but are not limited to, hybridizing at 68°C in 0.5M NaH_2PO4 (pH7.2)/1 mM EDTA/7% SDS, or hybridization in 50% formamide/0.25M NaH_2PO_4 (pH 7.2)/0.25 M NaC1/1 mM EDTA/7% SDS, followed by washing in 40 mM NaH_2PO_4 (pH 7.2)/1 mM EDTA/5% SDS at 50°C or in 40 mM NaH_2PO_4 (pH7.2)/ 1 mM EDTA/1% SDS at 50°C. Both temperature and salt can be varied, or alternatively, one or the other variable may remain constant while the other is changed.

Low, moderate and high stringency conditions are well known to those of skill in the art, and will vary predictably depending on the base composition of the particular nucleic acid sequence and on the specific organism from which the nucleic acid sequence is derived. For guidance regarding such conditions see, for example, Sambrook *et al. (supra)*; and Ausubel *et al. (supra)*.

RRN3 nucleic acids further include derivatives and analogs. Such derivatives and analogs can comprise at least one modified base moiety, such as, for example, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxy- hydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylamino-methyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v) , pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3- (3-amino-3-N-2-carboxypropyl) uracil, 2,6-diaminopurine, and the like. The *RRN3* nucleic acids can also have at least one modified sugar moiety, such as, for example, arabinose, 2-fluoroarabinose, xylulose, and hexose.

The *RRN3* nucleic acids can also have a modified phosphate backbone, such as, for example, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a

phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

The *RRN3* nucleic acids can also be an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (*see, e.g.*, Gautier *et al.*, *Nucl. Acids Res.* 15:6625-41 (1987)).

RRN3 nucleic acid derivatives or analogs can be synthesized by standard methods known in the art (*e.g.*, by use of a commercially available automated DNA synthesizer). As examples, phosphorothioate nucleic acids can be synthesized by the method of Stein *et al.* (*Nucl. Acids Res.* 16:3209-21 (1988)), and methyphosphonate nucleic acids oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. USA* 85:7448-51 (1988)), and the like.

Specific embodiments for the isolation of *RRN3* nucleic acids, presented as example but not by way of limitation, are as follows.

For expression cloning (a technique commonly known in the art), an expression library is constructed by methods known in the art. For example, mRNA (*e.g.*, human) is isolated, cDNA is prepared and then ligated into an expression vector (*e.g.*, a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed Rrn3 polypeptide. In one embodiment, anti-Rrn3 specific antibodies can be used for selection.

In another embodiment, polymerase chain reaction (PCR) can be used to amplify the desired sequence in a genomic or cDNA library, prior to selection. Oligonucleotide primers representing known *RRN3* sequences, for example, as selected from SEQ ID NO: 1, can be used as primers in PCR. In a typical embodiment, the oligonucleotide primers represent at least part of the *RRN3* conserved segments of strong identity between *RRN3* of different species. The synthetic oligonucleotides can be utilized as primers to amplify particular oligonucleotides within the *RRN3* gene by PCR sequences from a source (RNA or DNA), typically a cDNA library, of potential interest. PCR can be carried out, for example, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp). The DNA being amplified can include mRNA or cDNA or genomic DNA from any eukaryotic species. One of skill in the art can choose to

synthesize several different degenerate primers for use in the PCR reactions. For example, the CODEHOP strategy of Rose *et al.* (*Nucl. Acids Res.* 26:1628-35 (1998), which is incorporated by reference herein) can be used to design degenerate PCR primers using multiply-aligned sequences as a reference.

It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known *RRN3* nucleotide sequence and the related nucleic acid being isolated. For cross species hybridization, low stringency conditions are typically used. For same species hybridization, moderately stringent conditions are more typically used. After successful amplification of a segment of a related *RRN3* nucleic acid, that segment can be molecularly cloned and sequenced, and utilized as a probe to isolate a complete cDNA or genomic clone. This, in turn, can permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its polypeptide product for functional analysis, as described *infra*. In this fashion, additional genes encoding Rrn3 polypeptides and Rrn3 polypeptide derivatives can be identified.

The above-methods are not meant to limit the following general description of methods by which clones of *RRN3* nucleic acids or fragments can be obtained. Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the *RRN3* gene. The nucleic acid sequences encoding RRN3 can be isolated from vertebrate sources including, mammalian sources such as, porcine, bovine, feline, avian, equine, canine and human, as well as additional primate, avian, reptilian, amphibian, and piscine sources, and the like, from non-vertebrate sources, such as insects, worms, nematodes, plants, and the like. The DNA can be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (*See, e.g.*, Sambrook *et al., supra*; Glover, (ed.), *DNA Cloning: A Practical Approach*, IRL Press, Washington, DC. Vol. I, II. (1985).) Clones derived from genomic DNA can contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will typically contain only exon sequences. Whatever the source, the

nucleic acids can be molecularly cloned into a suitable vector for propagation of those nucleic acids.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode a *RRN3* gene. The DNA can be cleaved at specific sites using various restriction enzymes. Alternatively, one can use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific nucleic acid containing the desired gene can be accomplished in a number of ways. For example, a portion of a *RRN3* (of any species) gene or its specific RNA, or a fragment thereof can be purified and labeled. The generated DNA fragments can be screened by nucleic acid hybridization to the labeled probe (*see, e.g.*, Benton and Davis, *Science* 196:180-02 (1977); Grunstein and Hogness, *Proc. Natl. Acad. Sci. USA* 72:3961-65 (1975)). Those DNA fragments with substantial identity to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map, if such is available. Further selection can be carried out on the basis of the properties of the gene.

Alternatively, the presence of the *RRN3* nucleic acids can be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a polypeptide that, for example, has similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, promotion of cell proliferation activity, substrate binding activity, or antigenic properties as known for Rrn3 polypeptide(s). Immune serum or antibody which specifically binds to the Rrn3 polypeptide can be used to identify putatively Rrn3 polypeptide synthesizing clones by binding in an immunoassay, (e.g. an ELISA (enzyme-linked immunosorbent assay)-type procedure).

The *RRN3* gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to

isolate complementary mRNAs by hybridization. Such DNA fragments typically represent available, purified *RRN3* DNA of another species (*e.g.*, human, mouse, and the like). Immunoprecipitation analyses or functional assays (*e.g.*, stimulation of pol I transcription *in vitro*) of the *in vitro* translation products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs can be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against Rrn3 polypeptide. A radiolabeled *RRN3* cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA can then be used as a probe to identify the *RRN3* DNA from among other genomic DNA.

Alternatives to isolating the *RRN3* genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the Rrn3 polypeptide. For example, RNA for cDNA cloning of the *RRN3* gene can be isolated from cells that express the Rrn3 polypeptide. Other methods are possible and are considered within the scope of the invention.

The identified and isolated *RRN3* nucleic acids can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art can be used. Possible vectors include, but are not limited to, plasmids or modified viruses. The vector system is selected to be compatible with the host cell. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, yeast integrative and centromeric vectors, 2μ plasmid, and derivatives thereof, or plasmids such as pBR322, pUC, pcDNA3.1 or pRSET (Invitrogen) plasmid derivatives or the Bluescript vector (Stratagene), to name but a few. The insertion of the *RRN3* nucleic acids into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, however, the ends of the DNA molecules can be enzymatically modified. Alternatively, any desired restriction endonuclease site can be produced by ligating nucleotide sequences (*e.g.*, linkers) onto the DNA termini; these ligated sequences can comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and *RRN3* nucleic acids can be modified by

homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, and the like, so that many copies of the nucleic acid sequence are generated.

In an alternative method, the *RRN3* nucleic acids can be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the *RRN3* nucleic acids, for example, by size fractionation, can be done before insertion into the cloning vector. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated *RRN3* gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

Expression of the *RRN3* Gene:

The nucleotide sequence coding for a Rrn3 polypeptide, or a functionally active derivative, analog or fragment thereof, can be inserted into an appropriate expression vector (*i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted polypeptide-coding sequence). The necessary transcriptional and translational signals can also be supplied by the native *RRN3* gene and/or its flanking regions. A variety of host-vector systems can be utilized to express the polypeptide-coding sequence. These include, but are not limited to, mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, and the like), insect cell systems infected with virus (*e.g.*, baculovirus), microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used. In specific embodiments, the human *RRN3* gene is expressed, or a nucleic acid sequence encoding a functionally active portion of human Rrn3 is expressed in yeast or bacteria. In yet another embodiment, a fragment of *RRN3* comprising a domain of the Rrn3 polypeptide is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector can be used to construct expression vectors containing a chimeric

gene consisting of appropriate transcriptional/translational control signals and the polypeptide coding sequences. These methods include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequences encoding a Rrn3 polypeptide or fragment can be regulated by a second nucleic acid sequence so that the Rrn3 polypeptide or fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Rrn3 polypeptide can be controlled by any promoter/enhancer element known in the art. Promoters which can be used to control *RRN3* gene expression include, but are not limited to, the SV40 early promoter region (Benoist and Chambon, *Nature* 290:304-10 (1981)), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al.*, *Cell* 22:787-97 (1980)), the herpes thymidine kinase promoter (Wagner *et al.*, *Proc. Natl. Acad. Sci. USA* 78:1441-45 (1981)), the regulatory sequences of the metallothionein gene (Brinster *et al.*, *Nature* 296:39-42 (1982)), prokaryotic expression vectors such as the β-lactamase promoter (Villa-Komaroff *et al.*, *Proc. Natl. Acad. Sci. USA* 75:3727-31 (1978)) or the *tac* promoter (deBoer *et al.*, *Proc. Nalt. Acad. Sci. USA* 80:21-25 (1983)), plant expression vectors including the cauliflower mosaic virus 35S RNA promoter (Gardner *et al.*, *Nucl. Acids Res.* 9:2871-88 (1981)), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella *et al.*, *Nature* 310:115-20 (1984)), promoter elements from yeast or other fungi such as the *Gal7* and *Gal4* promoters, the ADH (alcohol dehydrogenase) promoter, the PGK (phosphoglycerol kinase) promoter, the alkaline phosphatase promoter, and the like.

The following animal transcriptional control regions, which exhibit tissue specificity, have been utilized for transgenic expression animals: the elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, *Cell* 38:639-46 (1984); Ornitz *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409 (1986); MacDonald, *Hepatology* 7(1 Suppl.):42S-51S (1987); the insulin gene control region which is active in pancreatic beta cells (Hanahan, *Nature* 315:115-22 (1985)), the immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, *Cell* 38:647-58 (1984); Adams *et al.*, *Nature* 318:533-8 (1985); Alexander *et al.*, *Mol. Cell. Biol.* 7:1436-44 (1987)), the mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, *Cell* 45:485-95 (1986)), the albumin gene control region which is active in liver (Pinkert *et al.*, *Genes Dev.* 1:268-

76 (1987)), the alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, *Mol. Cell. Biol.* 5:1639-48 (1985); Hammer *et al.*, *Science* 235:53-58 (1987)); the alpha 1- antitrypsin gene control region which is active in the liver (Kelsey *et al.*, *Genes and Devel.* 1:161-71 (1987)); the beta-globin gene control region which is active in myeloid cells (Magram *et al.*, *Nature* 315:338-40 (1985); Kollias *et al.*, *Cell* 46:89-94 (1986)); the myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead *et al.*, *Cell* 48:703-12 (1987)); the myosin light chain-2 gene control region which is active in skeletal muscle (Shani, *Nature* 314:283-86 (1985)); and the gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, *Science* 234:1372-78 (1986)).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a *RRN3*-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). For example, an expression construct can be made by subcloning a *RRN3* coding sequence into a restriction site of the pRSECT expression vector. Such a construct allows for the expression of the Rrn3 polypeptide under the control of the T7 promoter with a histidine amino terminal flag sequence for affinity purification of the expressed polypeptide.

Expression vectors containing *RRN3* nucleic acid inserts can be identified by general approaches well known to the skilled artisan, including: (a) nucleic acid hybridization, (b) the presence or absence of "marker" gene function, and (c) expression of inserted sequences. In the first approach, the presence of a *RRN3* nucleic acid inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted *RRN3* nucleic acid. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, and the like) caused by the insertion of a vector containing the *RRN3* nucleic acids. For example, if the *RRN3* nucleic acid is inserted within the marker gene sequence of the vector, recombinants containing the *RRN3* insert can be identified by the absence of marker gene function.

In the third approach, recombinant expression vectors can be identified by assaying the Rrn3 polypeptide expressed by the recombinant. Such assays can be based,

for example, on the physical or functional properties of the Rrn3 polypeptide in *in vitro* assay systems (*e.g.*, binding with anti-Rrn3 antibody, promotion of rRNA transcription, and the like). Once a particular recombinant DNA molecule is identified and isolated, several methods that are known in the art can be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain can be chosen that modulates the expression of the inserted sequences, or modifies or processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered Rrn3 polypeptide can be controlled. Furthermore, different host cells having characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation) of polypeptides can be used. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a mammalian protein. Furthermore, different vector/host expression systems can affect processing reactions to different extents.

Rrn3 Polypeptides, Fragments, Derivatives and Analogs:

The invention further relates to Rrn3 polypeptides, fragments, derivatives and analogs thereof. In one aspect, the invention provides amino acid sequences of Rrn3 polypeptide, typically human Rrn3 polypeptide (SEQ ID NO:2). In particular aspects, the polypeptides, fragments, derivatives, or analogs of Rrn3 polypeptides are from an animal (*e.g.*, human, mouse, rat, pig, cow, dog, monkey, and the like). The production and use of Rrn3 polypeptides, fragments, derivatives and analogs thereof are also within the scope of the present invention. In a specific embodiment, the fragment, derivative or analog is

functionally active (*i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type Rrn3 polypeptide). As one example, such fragments, derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of Rrn3 activity, and the like. Fragments, derivatives or analogs that retain, or alternatively lack or inhibit, a desired Rrn3 property of interest (*e.g.*, binding to a Rrn3 binding partner, stimulation of rRNA transcription, or modulation (*e.g.*, inhibition or stimulation) of cell proliferation) can be used as inducers, or inhibitors of such property and its physiological correlates. A specific embodiment relates to a Rrn3 fragment that can be bound by an anti-Rrn3 antibody. Fragments, derivatives or analogs of Rrn3 can be tested for the desired activity by procedures known in the art, including but not limited to the functional assays described herein.

Rrn3 polypeptide derivatives include naturally-occurring amino acid sequence variants as well as those altered by substitution, addition or deletion of one or more amino acid residues that provide for functionally active molecules. Rrn3 polypeptide derivatives include, but are not limited to, those containing as a primary amino acid sequence of all or part of the amino acid sequence of a Rrn3 polypeptide including altered sequences in which one or more functionally equivalent amino acid residues (*e.g.*, a conservative substitution) are substituted for residues within the sequence, resulting in a silent change.

In another aspect, Rrn3 polypeptides include those peptides having one or more consensus amino acid sequences shared by all Rrn3 family members, but not found in other proteins. These regions are conserved in both sequence and length and display the general consensus:

(1) Tyr(Ile/Leu)(Ala/Gly)(Ala/Ser)(Phe/Tyr)(Ile/Leu)(Ala/Ser)ArgAlaLys;

(2) PheTyr(Ala/Ser)XaaXaaGln(Ala/Ser)(Ile/Leu)XaaXaaXaa (Phe)XaaPheArg; and

(3) PhePro(Phe/Tyr)AspXaaXaaXaaLeu(Lys);

where parentheses indicate positions which vary among family members, and Xaa indicates variable positions. Database analysis indicates that these consensus sequences are not found in other polypeptides, and therefore this evolutionary conservation reflects the pol I-specific function of Rrn3 polypeptides. Rrn3 family members, including Rrn3

polypeptides, fragments, derivatives and/or analogs comprising one or more of these consensus sequences, are also within the scope of the invention. In a preferred embodiment, the Rrn3 family member is other than *Saccharomyces cerevisiae* Rrn3 polypeptide.

In another aspect, a polypeptide consisting of or comprising a fragment of a Rrn3 polypeptide having at least 10 contiguous amino acids of the Rrn3 polypeptide is provided. In other embodiments, the fragment consists of at least 20 or 50 contiguous amino acids of the Rrn3 polypeptide. In a specific embodiment, the fragments are not larger than 35, 100 or even 200 amino acids.

Fragments, derivatives or analogs of Rrn3 polypeptide include but are not limited to those molecules comprising regions that are substantially similar to Rrn3 polypeptide or fragments thereof (*e.g.*, in various embodiments, at least 50%, 60%, 70%, 75%, 80%, 90%, or even 95% identity or similarity over an amino acid sequence of identical size), or when compared to an aligned sequence in which the alignment is done by a computer sequence comparison/alignment program known in the art, or whose coding nucleic acid is capable of hybridizing to a *RRN3* nucleic acid, under high stringency, moderate stringency, or low stringency conditions (*supra*). Rrn3 polypeptides further comprise fragments and derivatives having an antigenic determinant (*e.g.*, can be recognized by an antibody specific for human Rrn3 polypeptide).

The Rrn3 polypeptide derivatives and analogs can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned *RRN3* nucleic acids can be modified by any of numerous strategies known in the art (*see, e.g.,* Sambrook *et al., supra*), such as making conservative substitutions, deletions, insertions, and the like. The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the *RRN3* nucleic acids encoding a fragment, derivative or analog of a Rrn3 polypeptide, the modified nucleic acid typically remains in the proper translational reading frame, so that the reading frame is not interrupted by translational stop signals or other signals which interfere with the synthesis of the Rrn3 fragment, derivative or analog. The *RRN3* nucleic acid can also be mutated *in vitro* or *in vivo* to create and/or destroy translation, initiation and/or termination sequences. The Rrn3 encoding nucleic acid can also be mutated to

create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones and to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchison *et al.*, *J. Biol. Chem.* 253:6551-60 (1978)), the use of TAB® linkers (Pharmacia), and the like.

Manipulations of the Rrn3 polypeptide sequence can also be made at the polypeptide level. Included within the scope of the invention are Rrn3 polypeptide fragments, derivatives or analogs which are differentially modified during or after synthesis (*e.g.*, *in vivo* or *in vitro* translation). Such modifications include conservative substitution, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, and the like. Any of numerous chemical modifications can be carried out by known techniques, including, but not limited to, specific chemical cleavage (*e.g.*, by cyanogen bromide), enzymatic cleavage (*e.g.*, by trypsin, chymotrypsin, papain, V8 protease, and the like); modification by, for example, $NaBH_4$ acetylation, formylation, oxidation and reduction, or metabolic synthesis in the presence of tunicamycin, and the like.

In addition, fragments, derivatives and analogs of Rrn3 polypeptides can be chemically synthesized. For example, a peptide corresponding to a portion, or fragment, of a Rrn3 polypeptide, which comprises a desired domain, or which mediates a desired activity *in vitro*, can be synthesized by use of chemical synthetic methods using, for example, an automated peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Rrn3 polypeptide sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ- amino butyric acid, ∈-Ahx, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, selenocysteine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, C α-methyl amino acids, N α-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the Rrn3 fragment or derivative is a chimeric, or fusion, protein comprising a Rrn3 polypeptide or fragment thereof (typically consisting of at least a domain or motif of the Rrn3 polypeptide, or at least 10 contiguous amino acids of the Rrn3 polypeptide) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein. The chimeric product can be made by ligating the appropriate nucleic acid sequence, encoding the desired amino acid sequences, to each other in the proper coding frame and expressing the chimeric product by methods commonly known in the art. Alternatively, the chimeric product can be made by protein synthetic techniques (*e.g.*, by use of an automated peptide synthesizer).

Rrn3 polypeptides can be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, sizing column chromatography, high pressure liquid chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties can be evaluated using any suitable assay as described herein or otherwise known to the skilled artisan. Alternatively, once a Rrn3 polypeptide produced by a recombinant is identified, the amino acid sequence of the polypeptide can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (*see*, *e.g.*, Hunkapiller *et al.*, *Nature* 310:105-11 (1984); Stewart and Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL, (1984)).

In another alternate embodiment, native Rrn3 polypeptides can be purified from natural sources by standard methods such as those described above (*e.g.*, immunoaffinity purification). In a specific embodiment of the present invention, Rrn3 polypeptides, whether produced by recombinant DNA techniques, by chemical synthetic methods or by purification of native polypeptides, include but are not limited to those containing as a primary amino acid sequence all or part of the amino acid sequence of human Rrn3 polypeptide (SEQ ID NO:2), as well as fragments, derivatives and analogs thereof.

Structure of the *RRN3* Gene and Polypeptide(s):

The structure of the *RRN3* gene and Rrn3 polypeptide can be analyzed by various methods known in the art. The cloned DNA or cDNA corresponding to the *RRN3* gene can be analyzed by methods including but not limited to Southern hybridization (Southern, *J. Mol. Biol.* 98:503-17 (1975)), Northern hybridization (*see, e.g.,* Freeman *et al., Proc. Natl. Acad. Sci. USA* 80:4094-98 (1983)), restriction endonuclease mapping (*see generally* Sambrook *et al.,supra*), and DNA sequence analysis (*see, e.g.*, Sambrook *et al., supra*). Polymerase chain reaction (PCR; *see, e.g.*, U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllensten *et al., Proc. Natl. Acad. Sci. USA* 85:7652-56 (1988); Ochman *et al., Genetics* 120:621-3 (1988); Loh *et al., Science* 243:217-20 (1989)) followed by Southern hybridization with a *RRN3*-specific probe can allow the detection of the *RRN3* gene in DNA from various cell types. Methods of amplification other than PCR are commonly known and can also be employed.

In one embodiment, Southern blot hybridization can be used to determine the genetic linkage of the *RRN3* locus. Northern blot hybridization analysis can be used to determine the expression of the *RRN3* gene. Various cell types at various states of development or activity can be tested for *RRN3* expression. The stringency of the hybridization conditions for both Southern and Northern blot hybridization can be manipulated to ensure detection of nucleic acids with the desired degree of sequence identity to the specific *RRN3* probe used. Modifications of these and other methods commonly known in the art can be used. Restriction endonuclease mapping can be used to roughly determine the genetic structure of the *RRN3* gene. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis. DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (*Meth. Enzymol.* 65:499-560 (1980)), the Sanger dideoxy method (Sanger *et al., Proc. Natl. Acad. Sci. USA* 74:5463-67 (1977)), the use of T7 DNA polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequencer (*e.g.*, Applied Biosystems, Foster City, CA).

The amino acid sequence of the Rrn3 polypeptide can be derived by deduction from the DNA sequence, or alternatively, by direct sequencing of the protein (*e.g.*, with an automated amino acid sequencer). The Rrn3 polypeptide sequence can be further characterized by a hydrophilicity analysis (Hopp and Woods, *Proc. Natl. Acad. Sci.*

USA 78:3824-28 (1981)). A hydrophilicity profile can be used to identify the hydrophobic and hydrophilic regions of the Rrn3 polypeptide and the corresponding regions of the gene sequence which encode such regions.

Secondary structural analysis (*e.g.,* Chou and Fasman, *Biochemistry* 13:222-45 (1974)) can also be conducted to identify regions of the Rrn3 polypeptide that assume specific secondary structures. Manipulation, translation, and secondary structure prediction, open reading frame prediction and plotting, as well as determination of sequence identity and similarities, can also be accomplished using computer software programs available in the art, such as those described above. Other methods of structural analysis can also be employed. These include but are not limited to X-ray crystallography (Engstom, *Biochem. Exp. Biol.* 11:7-13 (1974)) and computer modeling (Fletterick and Zoller, (eds.), "Computer Graphics and Molecular Modeling", In *Current Communications in Molecular Biology*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1986); Bordo, *Comput. Appl. Biosci.* 9:639-45 (1993); Bruccoleri and Karpus, *Biopolymers* 26:137-68 (1987); Hansen *et al. Pac. Symp. Biocomput.* 106-17 (1998)); Li *et al., Protein Sci.* 6:956-70 (1997); Sternberg and Zvelebil, *Eur. J. Cancer* 26:1163-66 (1990); Ring and Cohen, *FASEB J.* 7:783-90 (1993); and Sutcliffe *et al., Protein Eng.* 1:377-84 (1987)).

Antibodies to Rrn3 Polypeptides, Fragments, Derivatives and Analogs:

Rrn3 polypeptides, fragments, derivatives, and analogs thereof, can be used as an immunogen to generate antibodies which immunospecifically bind such Rrn3 polypeptides, fragments, derivatives, and analogs thereof. Such antibodies include but are not limited to polyclonal antibodies, monoclonal antibodies, chimeric antibodies, single chain antibodies, heavy chain antibody fragments (*e.g.*, F(ab'), $F(ab')_2$, Fv, or hypervariable regions), and an Fab expression library. In a specific embodiment, polyclonal and/or monoclonal antibodies to whole, intact human Rrn3 polypeptide are produced. In another embodiment, antibodies to a domain of a human Rrn3 polypeptide are produced. In another embodiment, fragments of a human Rrn3 polypeptide identified as hydrophilic are used as immunogens for antibody production.

Methods for making and using antibodies are generally disclosed by Harlow and Lane (Using Antibodies, A Laboratory Manual, Cold Spring Harbor

Laboratory, Cold Spring Harbor, New York (1999); the disclosure of which is incorporated by reference herein). Various procedures known in the art can be used for the production of polyclonal antibodies to a Rrn3 polypeptide, fragment, derivative or analog thereof. For the production of such antibodies, various host animals (including, but not limited to, rabbits, mice, rats, sheep, goats, camals, llamas and the like) can be immunized by injection with the native Rrn3 polypeptide, fragment, derivative or analog. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

For preparation of monoclonal antibodies directed toward a Rrn3 polypeptide, fragment, derivative, or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture can also be used. Such techniques include, for example, the hybridoma technique originally developed by Kohler and Milstein (*see, e.g., Nature* 256:495-97 (1975)), as well as the trioma technique, (*see, e.g.,* Hagiwara and Yuasa, Hum. Antibodies Hybridomas 4:15-19 (1993)), the human B-cell hybridoma technique (*see, e.g.*, Kozbor *et al., Immunology Today* 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (*see, e.g.*, Cole *et al.*, In *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)). Human antibodies can be used and can be obtained by using human hybridomas (*see, e.g.*, Cote *et al., Proc. Natl. Acad. Sci. USA* 80:2026-30 (1983)) or by transforming human B cells with EBV virus *in vitro* (*see, e.g.*, Cole *et al., supra*).

Further to the invention, "chimeric" or "humanized" antibodies (*see, e.g.*, Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-5 (1984); Neuberger *et al., Nature* 312:604-08 (1984); Takeda *et al., Nature* 314:452-4 (1985)) can be prepared. Such chimeric antibodies are typically prepared by splicing the non-human genes for an antibody molecule specific for a Rrn3 polypeptide together with genes from a human antibody molecule of appropriate biological activity. It can be desirable to transfer the antigen binding regions (*e.g.*, $F(ab')_2$, F(ab'), Fv, or hypervariable regions) of non-human antibodies into the framework of a human antibody by recombinant DNA techniques to

produce a substantially human molecule. Methods for producing such "chimeric" molecules are generally well known and described in, for example, U.S. Patent Nos. 4,816,567; 4,816,397; 5,693,762; and 5,712,120; International Patent Publications WO 87/02671 and WO 90/00616; and European Patent Publication EP 239 400; the disclosures of which are incorporated by reference herein). Alternatively, a human monoclonal antibody or portions thereof can be identified by first screening a human B-cell cDNA library for DNA molecules that encode antibodies that specifically bind to an Rrn3 polypeptide according to the method generally set forth by Huse *et al.* (*Science* 246:1275-81 (1989)). The DNA molecule can then be cloned and amplified to obtain sequences that encode the antibody (or binding domain) of the desired specificity. Phage display technology offers another technique for selecting antibodies that bind to Rrn3 polypeptides, fragments, derivatives or analogs thereof. (*See, e.g.*, International Patent Publications WO 91/17271 and WO 92/01047; and Huse *et al.*, *supra*).

According to another aspect of the invention, techniques described for the production of single chain antibodies (*see, e.g.*, U.S. Patent Nos. 4,946,778 and 5,969,108) can be adapted to produce Rrn3-specific single chain antibodies. An additional aspect of the invention utilizes the techniques described for the construction of a Fab expression library (*see, e.g.*, Huse *et al.* (1989) *supra*) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for Rrn3 polypeptides, fragments, derivatives, or analogs thereof.

The immunoglobulins also can be heavy chain antibodies. Immunoglobulins from animals such as camels, dromedaries, and llamas (Tylopoda) can form heavy chain antibodies, which comprise heavy chains without light chains. (See, e.g., Desmyter et al., J. Biol. Chem. 276:26285-90 (2001); Muyldermans and Lauwereys, J. Mol. Recognit. 12:131-40 (1999); Arbabi Ghahroudi et al., FEBS Lett. 414:521-26 (1997); Muyldermans et al., Protein Eng. 7:1129-35 (1994); Hamers-Casterman et al., Nature 363:446-48 (1993); the disclosures of which are incorporated by reference herein.) The variable region of heavy chain antibodies are typically referred to as "VHH" regions. (See, e.g., Muyldermans et al., TIBS 26:230-35 (2001).) The VHH of heavy chain antibodies typically have enlarged or altered CDR regions, as such enlarged CDR1 and/or CDR3 regions. Methods of producing heavy chain antibodies are also known in the art. (See, e.g., Arbabi Ghahroudi et al., supra; Muyldermans and Lauwereys, supra.)

Antibody which contains the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to, the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule, the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments. Recombinant Fv fragments can also be produced in eukaryotic cells using, for example, the methods described in U.S. Patent No. 5,965,405.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art (*e.g.*, ELISA (enzyme-linked immunosorbent assay)). In one example, antibodies which recognize a specific domain of a Rrn3 polypeptide can be used to assay generated hybridomas for a product which binds to a Rrn3 fragment containing that domain. For selection of an antibody that specifically binds to a first Rrn3 polypeptide derivative, but which does not specifically bind a different Rrn3 polypeptide, one can select on the basis of antibody positive binding to the first Rrn3 polypeptide and a lack of antibody binding to the second different Rrn3 polypeptide.

Antibodies specific to a domain of Rrn3 polypeptides are also provided. The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the Rrn3 polypeptide sequences of the invention (*e.g.*, for imaging proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, and the like). In another embodiment of the invention (*see infra*), anti-Rrn3 antibodies and fragments thereof containing the antigen-binding domain are used as agents and compositions to slow or abate the growth of proliferative disease.

Functional Assays for Rrn3 Polypeptides, Fragments, Derivatives, and Analogs:

The functional activity of Rrn3 polypeptides, fragments, derivatives and analogs can be assayed by various methods. For example, when assaying for the ability to bind or compete with wild-type Rrn3 polypeptide for binding to anti-Rrn3 antibody, various immunoassays known in the art can be used. Such assays include, but are not limited to, competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay) "sandwich"

immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, and the like), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, immunoelectrophoresis assays, and the like. (See generally Harlow and Lane, *supra*). Antibody binding can be detected by measuring a label on the primary antibody. Alternatively, the primary antibody is detected by measuring binding of a secondary antibody or reagent to the primary antibody. The secondary antibody can also be directly labeled. Many means are known in the art for detecting binding in an immunoassay and are considered within the scope of the present invention.

In another embodiment, the ability of Rrn3 polypeptide to stimulate rRNA transcription is assayed. For example, transcription stimulation can be measured by the method of Klein and Grummt (*Proc. Natl. Acad. Sci. USA* 96:6096-101 (1999). Briefly, standard transcription reactions (25 μl), containing 25 μg of a whole cell extract of protein from mammalian cells, include 25 ng of rRNA template (*e.g.*, the human or mouse rRNA sequences)/12 mM Tris-HCl, pH 7.9/0.1 mM EDTA/0.5 mM dithioerythritol/5 mM $MgCl_2$/80 mM KCl/12 % glycerol/0.66 each ATP, CTP, and GTP/0.01 mM UTP with 1 μCi of [α-^{32}P]UTP (3,000 Ci/mmol). Transcription is performed for 60 minutes at 30° C. After completion of transcription, the amount of transcription is quantitated by known methods, such as by separating the unincorporated radioactively labeled nucleotides from the labeled transcripts and then counting in a scintillation counter or by separation using gel electrophoresis. In such assays, the endogenous Rrn3 polypeptide can be depleted by anti-Rrn3 antibody.

In still another embodiment, the functional activity of a Rrn3 polypeptide, fragment, derivative, or analog is determined in an *in vivo* system. For example, stimulation of rRNA transcription or physiological changes in cells or tissues can be measured and correlated with Rrn3 activity by expressing *RRN3* nucleic acids in mammalian cells and examining the effect on cell proliferation. Alternatively, Rrn3 polypeptides, fragments, derivatives, or analogs can be expressed in a heterologous system and the activity of the Rrn3 polypeptide, fragment, derivative or analog assayed as a physiological changes in that system. For example, the ability of a Rrn3 polypeptide to

stimulate rRNA transcription *in vivo* can be tested using the yeast plasmid shuffling system. In particular, a yeast strain that has a null allele of the endogenous *RRN3* gene can be rescued by an extrachromosomal copy of the yeast *RRN3* gene, or a heterologous *RRN3* nucleic acid from another eukaryotic organism. For example, the ability of the heterologous (*e.g.*, human) *RRN3* nucleic acid to complement the yeast *RRN3* null allele can be determined. Similarly, the activity of a Rrn3 fragment, derivative or analog can be determined by the same complementation assay. The activity of mutant *RRN3* nucleic acids can also be determined. Other eukaryotic *RRN3* genes can also be isolated by complementation of the yeast null allele, as will be appreciated by the skilled artisan.

In Vivo Uses of *RRN3* Nucleic Acids, Rrn3 Polypeptides, Fragments, Derivatives, Analogs and Antibodies:

The invention provides further for methods for the administration of one or more agents, or compositions containing such agents, which modulate cell proliferation. Such agents include, but are not limited to, Rrn3 polypeptides, fragments, derivatives and analogs thereof as described hereinabove; antibodies specific for Rrn3 polypeptide, fragments, derivatives and analogs thereof (as described hereinabove); nucleic acids encoding Rrn3 polypeptides, fragments, derivatives and analogs thereof (as described hereinabove); *RRN3* antisense nucleic acids, and Rrn3 polypeptide agonists and antagonists. The Rrn3 agents can be used to treat disorders involving cell hyperproliferation (*e.g.*, tumorigenesis) or hypoproliferation by altering Rrn3 function.

Generally, it is typical to administer an agent of a species origin or species reactivity (in the case of antibodies) that is the same as that of the recipient. Thus, a human Rrn3 polypeptide, fragment, derivative, or analog thereof, or *RRN3* nucleic acid or fragment or analog thereof, or an antibody to a human Rrn3 polypeptide, is administered to a human in a dose which is therapeutically or prophylactically effective.

Diseases involving cell hyperproliferation are treated or prevented, for example, by administration of an agent that decreases Rrn3 function. Examples of such an agent include, but are not limited to, anti-sense *RRN3* nucleic acids under the control of a strong inducible promoter, particularly those that are active in inhibiting cell proliferation. Other agents that can be used to decrease Rrn3 activity include anti-Rrn3 antibodies, or

those that can be identified using *in vitro* assays or animal models, examples of which are described herein.

In specific embodiments, agents that decrease *RRN3* function are administered therapeutically (including prophylactically) in diseases involving an increased (relative to normal or desired) level of Rrn3 polypeptide or function. For example, the agent can be administered to a patient where Rrn3 polypeptide is overexpressed, genetically defective, or biologically hyperactive, as compared with a normal cell of that type. Further, an agent of the invention can be administered in diseases or disorders wherein *in vitro* (or *in vivo*) assays indicate the utility of Rrn3 antagonist administration. For example, *RRN3* gene function can be specifically targeted to cancer cells to kill those cells.

The level in Rrn3 polypeptide or function can be detected, for example, by obtaining a patient tissue sample (such as from a biopsy tissue) and assaying it *in vitro* for RNA or polypeptide levels, structure and/or activity of the expressed *RRN3* RNA or Rrn3 polypeptide. Many methods standard in the art can be thus employed including, but not limited to, immunoassays to detect and/or visualize Rrn3 polypeptide (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis ("SDS PAGE"), immunocytochemistry, and the like) and/or hybridization assays to detect *RRN3* expression by detecting and/or visualizing *RRN3* mRNA (*e.g.*, Northern blot assays, dot blots, *in situ* hybridization, quantitative reverse transcriptase-PCR, and the like), among others known to the skilled artisan.

Diseases involving cell hyperproliferation that can be treated or prevented include but are not limited to malignancies, premalignant conditions (*e.g.*, hyperplasia, metaplasia, dysplasia), benign tumors, hyperproliferative disorders, benign dysproliferative disorders, and the like. In specific embodiment, an agent of the invention is administered to a human patient to prevent progression to cancer. Diseases involving cell hypoproliferation that can be treated or prevented include but are not limited to cardiac disease and other conditions in which an increase in cell proliferation is desired.

Gene Therapy:

RRN3 nucleic acids of the present invention can be used in the process of gene therapy. Gene therapy refers to the process of providing for the expression of nucleic

acid sequences of exogenous origin in a subject for the treatment of a disease within that subject. In a specific embodiment, anti-sense nucleic acids complementary to a sequence encoding a Rrn3 polypeptide, fragment, derivative or analog thereof, are administered to inhibit *RRN3* gene function, by way of gene therapy. In another embodiment, nucleic acids encoding Rrn3 polypeptide, or a fragment, derivative or analog thereof, are administered to repair a defective *RRN3* gene, or to stimulate *RRN3* gene activity. Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel *et al.* (*Clin. Pharm.* 12:488-505 (1993)); Wu and Wu (*Biotherapy* 3:87-95 (1991)); Tolstoshev (*Ann. Rev. Pharmacol. Toxicol.* 32:573-96 (1993)); Mulligan (*Science* 260:926-32 (1993)); Morgan and Anderson (*Ann. Rev. Biochem.* 62:191-217 (1993)); and May (*TIBTECH* 11:155-215 (1993)).

Methods commonly known in the art of recombinant DNA technology that can be used include those described in Ausubel *et al. (supra)* and Kriegler (*Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY (1990)). In one embodiment, the agent comprises a *RRN3* sense nucleic acid that is part of an expression vector that expresses a Rrn3 polypeptide or fragment or chimeric protein thereof in a suitable host cell. In particular, such a nucleic acid has a promoter operably linked to the *RRN3* coding region, the promoter being inducible or constitutive, and, optionally, tissue-specific. In another embodiment, the agent comprises a *RRN3* antisense nucleic acid that is part of an expression vector that expresses the antisense nucleic acid in a suitable host. In particular, such an antisense nucleic acid has a promoter operably linked to the *RRN3* antisense nucleic acid, the promoter being inducible or constitutive, and, optionally, tissue-specific.

In another particular embodiment, a nucleic acid is used in which the *RRN3* coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the *RRN3* nucleic acid (*see, e.g.,* Koller and Smithies, *Proc. Natl. Acad. Sci. USA* 86:8932-35 (1989); Zijlstra *et al.*, *Nature* 342:435-38 (1989); U.S. Patent Nos. 5,631,153; 5,627,059; 5,487,992; and 5,464,764)). *RRN3* nucleic acids that are dysfunctional (*e.g.*, due to a heterologous (non-*RRN3* sequence) insertion within the *RRN3* coding sequence) can be used to "knockout" endogenous *RRN3* function by

homologous recombination (*see, e.g.,* Capecchi, *Science* 244:1288-92 (1989); U.S. Patent Nos. 5,631,153; 5,627,059; 5,487,992; and 5,464,764)). In a specific embodiment of the invention, a nucleic acid containing a portion of a *RRN3* gene, in which the *RRN3* sequences flank, both 5' and 3', a different gene sequence, is used to disrupt the expression of an *RRN3* gene by homologous recombination (*see* Koller and Smithies, *(supra)*; Zijlstra *et al. (supra)*.

For any of these embodiments, delivery of the nucleic acid into a patient can be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy. In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art (*e.g.,* by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, for example, by infection using a defective or attenuated retroviral or other viral vector (*see, e.g.*, U.S. Patent No. 4,980,286), by direct injection of naked DNA, or by use of microparticle bombardment, such as a gene gun (BIOLISTIC™, Dupont). DNA can also be inserted into cells by coating naked DNA with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering the DNA in linkage to a peptide which is known to enter the nucleus, by administering the DNA in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, *J. Biol. Chem.* 262:4429-32 (1987)), which can be used to target cell types specifically expressing the receptors, and the like. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation.

In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, International Patent Publications WO 92/06180; WO 92/22635; WO 92/20316; WO 93/14188; and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination (*see, e.g.*,

Koller and Smithies *supra*; Zijlstra *et al. supra*; U.S. Patent Nos. 5,631,153; 5,627,059; 5,487,992; and 5,464,764).

In a specific embodiment, a viral vector is used that contains the *RRN3* nucleic acid (sense or antisense). For example, a retroviral vector can be used (*see* Miller *et al.*, *Meth. Enzymol.* 217:581-99 (1993)). These retroviral vectors are typically modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The *RRN3* nucleic acid to be used in gene therapy is cloned into the vector, which facilitates delivery of the nucleic acid into a patient. More detail about retroviral vectors can be found in Boesen *et al.* (*Biotherapy* 6:291-302 (1994)), which describes the use of a retroviral vector to deliver the *mdrl* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Lentiviral vectors can also be used. (*See, e.g.*, Naldini *et al.*, *Science* 272:263-67 (1996).) Other references illustrating the use of viral vectors in gene therapy are: Clowes *et al.* (*J. Clin. Invest.* 93:644-51 (1994)); Kiem *et al.* (*Blood* 83:1467-73 (1994)); Salmons and Gunzberg (*Hum Gene Ther.* 4:129-41 (1993)); and Grossman and Wilson (*Curr. Opin. Genet Dev.* 3:110-14 (1993)).

Adenoviruses can also be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are prostate, liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson (*Curr. Opin. Genet Dev.* 3:499-503 (1993)) present a review of adenovirus-based gene therapy. Bout *et al.* (*Human Gene Therapy* 5:3-10 (1994)) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Herman *et al.* (*Human Gene Therapy* 10:1239-49 (1999)) describe the intraprostatic injection of a replication-deficient adenovirus containing the herpes simplex thymidine kinase gene into human prostate, followed by intravenous administration of the prodrug ganciclovir in a phase I clinical trial. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.* (*Science* 252:431-34 (1991)); Rosenfeld *et al.* (*Cell* 68:143-55 (1992)); Mastrangeli *et al.* (*J. Clin. Invest.* 91:225-34 (1993)); and Thompson (*Oncol. Res* 11:1-8 (1999)). Adeno-associated virus (AAV) can also be used in gene therapy (*see, e.g.*, Ali *et al.*, *Gene*

Therapy 1:367-84 (1994); U.S. Patent Nos. 4,797,368 and 5,139,941; Walsh *et al.*, *Proc. Soc. Exp. Biol. Med.* 204:289-300 (1993); Grimm *et al.*, *Human Gene Therapy* 10:2445-50 (1999)).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by methods such as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Typically, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. The selected cells are then delivered to a patient.

In one embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, and the like. Numerous techniques are known in the art for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, *Meth. Enzymol.* 217:599-618 (1993); Cotten *et al.*, *Meth. Enzymol.* 217:618-44 (1993); Cline, *Pharmacol. Ther.* 29:69-92 (1985)) and can be used in accordance with the present invention. The technique typically provides for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and is heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Typically, cells are injected subcutaneously. In another embodiment, recombinant skin cells can be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are typically administered intravenously. The amount of cells required for use depends on the desired effect, the patient's condition, and the like, and can be determined by one skilled in the art.

Cells into which a *RRN3* nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to breast cells, prostate cells, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes, blood cells (such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes), and various stem or progenitor cells (in particular, hematopoietic stem or progenitor cells, such as those

obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, and the like). The cells used for gene therapy generally are autologous to the patient, but heterologous cells that can be typed for compatibility with the patient can be used.

Antisense Regulation of *RRN3* Expression:

In other embodiments, Rrn3 function is inhibited by use of *RRN3* antisense nucleic acids. The present invention provides for the administration of nucleic acids of at least six nucleotides that are antisense to a gene or cDNA encoding Rrn3, or a portion thereof, to inhibit the function of Rrn3 polypeptide. A *RRN3* "antisense" nucleic acid as used herein refers to a nucleic acid which hybridizes to a portion of a *RRN3* RNA (typically mRNA) by virtue of some sequence complementarity. The antisense nucleic acid can be complementary to a coding and/or noncoding region of a *RRN3* mRNA. Such antisense nucleic acids have utility as agents that inhibit Rrn3 function, and can be used in the treatment or prevention of disorders, as described *supra*.

The antisense nucleic acids can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA, or a derivative or analog thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced nucleic acid sequences.

In a specific embodiment, the *RRN3* antisense nucleic acid provided by the instant invention can be used to prevent tumor or other forms of aberrant cell proliferation. The invention further provides pharmaceutical compositions comprising an effective amount of the *RRN3* antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*. In another embodiment, the invention is directed to methods for inhibiting the expression of a *RRN3* nucleic acid sequence in a eukaryotic cell comprising providing the cell with an effective amount of a composition comprising a *RRN3* antisense nucleic acid. *RRN3* antisense nucleic acids and their uses are described in detail below.

RRN3 Antisense Nucleic Acids:

The *RRN3* antisense nucleic acids are of at least six nucleotides and are typically oligonucleotides (ranging from 6 to about 50 nucleotides or more). In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100

nucleotides, or can be at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or analogs thereof, and can be single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, and/or phosphate backbone. The oligonucleotide can include other appending groups such as peptides, or agents facilitating transport across the cell membrane (*see, e.g.*, Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA* 86:6553-56 (1989); Lemaitre *et al.*, *Proc. Natl. Acad. Sci. USA* 84:648-52 (1987); International Patent Publication WO 88/09810) or blood-brain barrier (*see, e.g.*, International Patent Publication WO 89/10134), hybridization-triggered cleavage agents (*see, e.g.*, Krol *et al.*, *BioTechniques* 6:958-76 (1988)) or intercalating agents (*see, e.g.*, Zon, *Pharm. Res.* 5:539-49 (1988)).

In one embodiment of the invention, a *RRN3* antisense oligonucleotide is provided, typically as single-stranded DNA. The oligonucleotide can be modified at any position on its structure with substituents generally known in the art. The *RRN3* antisense oligonucleotide can comprise at least one modified base moiety, such as, for example, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxy-hydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylamino-methyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N-6-isopentenyladenine, uracil-5-oxyacetic acid (v) , pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, 2,6-diaminopurine, and the like. In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, such as, for example, arabinose, 2-fluoroarabinose, xylulose, or hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone, such as, for example, a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (*see* Gautier *et al.*, *Nucl. Acids Res.* 15:6625-41 (1987)). The oligonucleotide can be conjugated to another molecule (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, and the like).

Antisense oligonucleotides of the invention can be synthesized by standard methods known in the art (*e.g.*, by use of a commercially available automated DNA synthesizer). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein *et al.* (*Nucl. Acids Res.* 16:3209-21 (1988)), methyphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. USA* 85:7448-51 (1988)), and the like.

In a specific embodiment, the *RRN3* antisense oligonucleotide comprises catalytic RNA, or a ribozyme (*see, e.g.*, International Patent Publication WO 90/11364; Sarver *et al.*, *Science* 247:1222-25 (1990)). In another embodiment, the oligonucleotide is a 2'-0-methylribonucleotide (Inoue *et al.*, *Nucl. Acids Res.* 15:6131-48 (1987)), or a chimeric RNA-DNA analogue (Inoue *et al.*, *FEBS Lett.* 215:327-30 (1987)).

In another specific embodiment, double-stranded RNA directs the sequence-specific degradation of mRNA by RNA interference. (*See generally* Hunter, *Curr. Biol.* 10:R137-40 (2000); Bosher and Labouesse, *Nat. Cell. Biol.* 2:e31-36 (2000).) Briefly, double-stranded *RRN3* nucleic acids are introduced into a cell to selectively inhibit *RRN3* gene expression by causing degradation of the *RRN3* mRNA. (*See, e.g.*, Zamore *et al.*, *Cell* 101:25-33 (2000).)

In an alternative embodiment, the *RRN3* antisense nucleic acid of the invention is produced intracellularly by gene therapy (*supra*). For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. The vector would contain a sequence encoding the *RRN3* antisense nucleic acid or a portion thereof. Once inside the cell the vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods

standard in the art. Vectors can be plasmid, viral, or others known in the art and used for replication and expression in mammalian cells. Expression of the nucleic acid encoding the *RRN3* antisense RNA can be controlled by any promoter known in the art to act in mammalian, typically human, cells. The promoters can be inducible or constitutive. Inducible promoters include but are not limited to, the SV40 early promoter region (Benoist and Chambon *supra*), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto *et al. supra*), the herpes thymidine kinase promoter (Wagner *et al. supra*), the regulatory sequences of the metallothionein gene (Brinster *et al. supra*), and the like.

Use of *RRN3* Antisense Nucleic Acids:

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *RRN3* gene, such as a human *RRN3* gene. Absolute complementarity, although typical, is not required, however. A sequence "complementary to at least a portion of an RNA," as used herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex. In the case of double-stranded *RRN3* antisense nucleic acids, a single strand of the duplex DNA can be tested, or triplex formation can be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches it can contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

The *RRN3* antisense nucleic acids can be administered to an individual to treat (or prevent) diseases of a cell type that expresses or overexpresses Rrn3 polypeptide or as a method to selectively inactivate certain cells by inhibiting production of Rrn3 polypeptide. In a specific embodiment, such a disease is hyperproliferation, such as cancer or tumor formation. In one embodiment, a single-stranded DNA antisense *RRN3* oligonucleotide is used. Cell types which express or overexpress *RRN3* RNA can be identified by various methods known in the art. Additionally, cell types which exhibit hyperproliferation due to other defects can also be identified by various methods known in the art. Such methods include, but are not limited to, hybridization with a *RRN3*-specific

nucleic acid (*e.g.*, by Northern blot hybridization, dot blot hybridization or *in situ* hybridization), observing the ability of RNA from the cell to be translated *in vitro* into Rrn3 polypeptide, immunoassay, and the like. In a typical example, primary tissue from a patient can be assayed for *RRN3* expression prior to treatment, for example, by immunocytochemistry or *in situ* hybridization. Similarly, methods for detecting hyperproliferation due to over- or under-expression of other genes can also be identified by various methods known in the art. *RRN3* antisense nucleic acids can be used to treat any of these diseases.

Compositions of the invention, including an effective amount of a *RRN3* antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a disease which is of a type that expresses or overexpresses *RRN3* RNA or Rrn3 polypeptide. The amount of *RRN3* antisense nucleic acid which will be effective in the treatment of a particular disease will depend on the nature of the disease, and can be determined by standard clinical techniques. Where possible, it is typical to determine the antisense cytotoxicity of the cell type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, compositions comprising *RRN3* antisense nucleic acids and a pharmaceutically acceptable carrier are administered via liposomes, microparticles, or microcapsules, as well as any other delivery modes described herein. In various embodiments of the invention, it can be useful to use such compositions to achieve sustained release of the *RRN3* antisense nucleic acids. In a specific embodiment liposomes targeted via antibodies to specific identifiable tumor antigens are utilized (*see, e.g.*, Leonetti *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2448-51 (1990); Renneisen *et al.*, *J. Biol. Chem.* 265:16337-42 (1990)).

Treatment and Prevention of Hyperproliferative and Dysproliferative Disorders:

Diseases involving an increase in cell proliferation (growth) or in which cell proliferation is otherwise undesirable, are also treated or prevented by administration of an agent that antagonizes (inhibits) Rrn3 function. In particular, because Rrn3 polypeptide is required for rRNA transcription, agents that interfere with *RRN3* gene expression or Rrn3 polypeptide levels or function can be used to treat such diseases. In addition to the agents described above, other agents that can be used further include, but

are not limited to, anti-Rrn3 antibodies (and fragments and derivatives thereof containing the antigen binding region thereof). Agents that inhibit Rrn3 function can be identified by use of known convenient *in vitro* assays (*e.g.*, based on their ability to inhibit binding of Rrn3 polypeptide to another protein or to inhibit any known Rrn3 function, as typically assayed *in vitro* or in cell culture) can also be employed. Typically, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific agent and whether its administration is indicated for treatment of the affected tissue.

Treatment and Prevention of Hypoproliferative Diseases:

Diseases involving decreased cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by stimulating Rrn3 activity, include but are not limited to degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds (for example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues), and the like. Such diseases can be treated by any of the methods described herein that increase Rrn3 activity or cell proliferation

Administration of Agents and Compositions:

The invention provides methods for the administration to a subject of an effective amount of an agent of the invention. Typically, the agent is substantially purified prior to formulation. The subject can be an animal, including but not limited to, cows, pigs, horses, chickens, cats, dogs, and the like, and is typically a mammal, and in a particular embodiment human. In another specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the agent comprises a nucleic acid are described above; additional appropriate formulations and routes of administration can be selected from among those described hereinbelow.

Various delivery systems are known and can be used to administer an agent, such as, for example, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the agent, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, *J. Biol. Chem.* 262:4429-32 (1987)), construction of an agent

comprising a nucleic acid as part of a retroviral or other vector, and the like. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes. The agents can be administered by any convenient route such as, for example, by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa), and the like, and can be administered together with other functionally active agents. Administration can be systemic or local. In addition, it can be desirable to introduce an agent into the target tissue by any suitable route, including intravenous and intrathecal injection. Pulmonary administration can also be employed, such as, for example, by use of an inhaler or nebulizer, and formulation of the agent with an aerosolizing agent.

In a specific embodiment, it can be desirable to administer the agent locally to the area in need of treatment; this administration can be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application (*e.g.*, in conjunction with a wound dressing after surgery), by injection, by means of a catheter, by means of a suppository, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre- neoplastic tissue.

In another embodiment, the agent can be delivered in a vesicle, in particular a liposome (*see, e.g.*, Langer, *Science* 249:1527-33 (1990); Treat *et al.*, In, *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-65 (1989); Lopez-Berestein, *supra*, pp. 317-27).

In yet another embodiment, the agent can be delivered in a controlled release system. In one embodiment, a pump can be used (*see, e.g.*, Langer, *supra*; Sefton, *Crit. Ref. Biomed. Eng.* 14:201-40 (1987); Buchwald *et al.*, *Surgery* 88:507-16 (1980); Saudek *et al.*, *N. Engl. J. Med.* 321:574-79 (1989)). In another embodiment, polymeric materials can be used (*see Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61- (1983); *see also* Levy *et al.*, *Science* 228:190-92 (1985); During *et al.*, *Ann. Neurol.* 25:351-56 (1989);

Howard *et al.*, *J. Neurosurg.* 71:105-12 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, in *Medical Applications of Controlled Release*, *supra*, Vol. 2, pp. 115-38 (1984)). Other controlled release systems are discussed in, for example, the review by Langer (*Science* 249:1527-33 (1990)).

Where the agent is a nucleic acid encoding a Rrn3 polypeptide, the nucleic acid can be administered *in vivo* to promote expression of its encoded polypeptide, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, for example, by use of a retroviral vector (*see* U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; BIOLISTIC™, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (*see, e.g.,* Joliot *et al.*, *Proc. Natl. Acad. Sci. USA* 88:1864-68 (1991)), and the like. Other modes of *in vivo* and *ex vivo* administration are described *supra*. Alternatively, an agent which comprises a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination as described above.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of an agent, and a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more typically in humans. The term "carrier" refers to a diluent, adjuvant, excipient, stabilizer, or vehicle with which the agent is formulated for administration. Pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is a typical carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition, if desired, can

also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Pharmaceutical compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations, and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. Examples of suitable pharmaceutical carriers are described in, for example, *Remington's Pharmaceutical Sciences* (Gennaro (ed.), Mack Publishing Co., Easton, Pennsylvania (1990)). Such compositions will contain a therapeutically effective amount of the agent, typically in purified form, together with a suitable amount of carrier so as to provide a formulation proper for administration to the patient. The formulation should suit the mode of administration.

In one embodiment, the agent is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition can also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form. For example, as a dry lyophilized powder or water-free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients can be mixed prior to administration.

The agents of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, and the like, and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The amount of the agent which will be effective in the treatment of a particular disease as indicated by modulation of cell proliferation will depend on the nature of the disease, and can be determined by standard clinical techniques. In addition, *in vitro* assays can optionally be employed to help identify optimal dosage ranges. The precise dose of the agent to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. Suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active agent per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses can be extrapolated from dose response curves derived from *in vitro* or animal model test systems. Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations typically contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Diagnosis and Screening:

Rrn3 polypeptides and *RRN3* nucleic acids, and fragments, derivatives, and analogs thereof, and anti-Rrn3 antibodies, also have utility in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various diseases (including conditions and disorders) affecting *RRN3* expression, or to monitor the treatment thereof. In particular, methods, such as an immunoassay, can be carried out by steps comprising contacting a sample derived from a patient with an anti-Rrn3 antibody under conditions conducive to immunospecific binding, and detecting or measuring the amount of any immunospecific binding by the antibody. In a particular aspect, binding of antibody to Rrn3 polypeptide, in tissue sections, can be used to detect aberrant Rrn3 localization or aberrant (*e.g.*, low, absent or elevated) levels of Rrn3 polypeptide. In a specific embodiment, antibody to Rrn3 polypeptide can be used to assay

a patient tissue or serum sample for the presence of Rrn3, where an aberrant level of Rrn3 is an indication of a disease. By "aberrant levels" is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disease.

The immunoassays which can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as Western blot, radioimmunoassay, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassay, immunoprecipitation assay, precipitin reaction, gel diffusion precipitin reaction, immunodiffusion assay, agglutination assay, complement-fixation assay, immunoradiometric assay, fluorescent immunoassay, protein A immunoassay, and the like.

RRN3 genes and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. *RRN3* nucleic acid sequences (*e.g.*, SEQ ID NO:1), or fragments thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor disease (including conditions and disorders) associated with aberrant changes in *RRN3* expression and/or activity, as described *supra*. In particular, a hybridization assay is carried out by a method comprising contacting a sample containing polynucleotides with a nucleic acid probe capable of hybridizing to *RRN3* DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

In specific embodiments, diseases involving hypo- or hyper-proliferation of cells can be diagnosed, or their suspected presence can be screened for, or a predisposition to develop such diseases can be identified by detecting decreased or increased levels of Rrn3 polypeptide, *RRN3* RNA, or Rrn3 functional activity. Additionally, hypo- or hyper-proliferation can be diagnosed by detecting mutations in *RRN3* RNA or DNA or Rrn3 polypeptide (*e.g.*, translocations in *RRN3* nucleic acids, truncations in the *RRN3* gene or Rrn3 polypeptide, changes in nucleotide or amino acid sequence relative to wild-type *RRN3,* or Rrn3, respectively) that cause decreased or increased expression or activity of Rrn3 polypeptide.

By way of example, levels of Rrn3 polypeptide in a biopsy can be detected by immunoassay; levels of *RRN3* RNA can be detected by hybridization assays (*e.g.*,

Northern blot or dot blot). Translocations and point mutations in *RRN3* nucleic acids can be detected by Southern blot, RFLP analysis, SSCP analysis, PCR using primers that typically generate a fragment spanning at least most of the *RRN3* gene, sequencing of the *RRN3* genomic DNA or cDNA obtained from the sample, and the like.

In one embodiment, levels of *RRN3* mRNA or Rrn3 polypeptide in a sample of a tissue isolated from a patient are detected or measured, in which increased levels indicate that the subject has, or has a predisposition to developing, a malignancy or hyperproliferative disease of that tissue, and in which the increased levels are relative to the levels present in an analogous sample from a portion of the body or from a subject not having the malignancy or other hyperproliferative disease, as the case may be.

In another specific embodiment, diseases involving a deficiency in cell proliferation or in which cell proliferation is desirable for treatment, are diagnosed, or their suspected presence can be screened for, or a predisposition to develop such diseases can be detected, by detecting decreased levels of Rrn3 polypeptide or *RRN3* mRNA. Additionally, a deficiency in cell proliferation can be diagnosed by detecting Rrn3 functional activity, or by detecting mutations in *RRN3* RNA or DNA or Rrn3 polypeptide (for example, translocations in *RRN3* nucleic acids, truncations in the gene or polypeptide, changes in nucleotide or amino acid sequence relative to wild-type *RRN3* gene or Rrn3 polypeptide) that cause decreased expression or activity of Rrn3. By way of example, levels of Rrn3 polypeptide, levels of *RRN3* mRNA, Rrn3 binding activity, and the presence of translocations or point mutations in the *RRN3* gene can be determined as described above.

In a specific embodiment, levels of *RRN3* mRNA or Rrn3 polypeptide in a patient sample are detected or measured, in which decreased levels indicate that the subject has, or has a predisposition to developing, a hypoproliferative disorder, in which the decreased levels are relative to the levels present in an analogous sample from a portion of the body or from a subject not having the hypoproliferative disorder, as the case may be.

Kits for diagnostic use are also provided that include, in one or more containers, an anti-Rrn3 antibody and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-Rrn3 antibody can be labeled with a detectable marker (*e.g.*, a chemiluminescent, enzymatic, fluorescent, radioactive moiety, and the like). A kit

is also provided that includes, in one or more containers, a nucleic acid probe capable of hybridizing to *RRN3* mRNA.

In a specific embodiment, a kit can include, in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides or more) that are capable of priming amplification (*e.g.*, by polymerase chain reaction (*see, e.g.*, Innis *et al.*, *PCR Protocols*, Academic Press, Inc., San Diego, CA (1989)), ligase chain reaction (*see, e.g.*, EP 320 308), use of Qβ replicase, cyclic 5' probe reaction, or other methods known in the art) under appropriate reaction conditions such that at least a portion of a *RRN3* nucleic acid is amplified. A kit can optionally further comprise in a container a predetermined amount of a purified Rrn3 polypeptide or *RRN3* nucleic acid, for example, for use as a standard or control.

Screening for Agonists and Antagonists:

RRN3 nucleic acids, Rrn3 polypeptide, and fragments, derivatives and analogs thereof, also have uses in screening assays to detect candidate compounds that specifically bind to *RRN3* nucleic acids, Rrn3 polypeptides, or fragments, derivatives or analogs thereof, and thus have use as agonists or antagonists. The agonists and antagonists can be identified by *in vitro* and/or *in vivo* assays. Such assays can be used to identify agents that are therapeutically effective, such as anti-proliferative agents, or as lead compounds for drug development. The invention thus provides assays to detect candidate compounds that specifically affect the activity or expression of *RRN3* nucleic acids, Rrn3 polypeptides, or fragments, derivatives or analogs thereof.

In a typical *in vivo* assay, recombinant cells expressing *RRN3* nucleic acids can be used to screen candidate compounds for those that affect *RRN3* expression. Effects on *RRN3* expression can include transcription of mRNA, translation of the mRNA, synthesis of Rrn3 polypeptides, effects on Rrn3 polypeptide function (*e.g.*, rRNA synthesis) and on Rrn3 polypeptide stability or localization. Such effects on *RRN3* expression can be identified as physiological changes, such as, for example, changes in cell growth rate, division or viability. In one embodiment, candidate compounds are administered to recombinant cells expressing Rrn3 polypeptide to identify those compounds that produce a physiological change. In another embodiment, the method comprises administering a candidate compound to a first cell that expresses a first Rrn3

polypeptide; administering the candidate compound to a second cell that expresses a second Rrn3 polypeptide; and determining whether the candidate compound modulates the activity of the first Rrn3 polypeptide but not the activity of the second Rrn3 polypeptide. For example, the first Rrn3 polypeptide can be yeast Rrn3 polypeptide and the second can be human Rrn3 polypeptide. Alternatively, the first Rrn3 polypeptide can be a mutant, and the second Rrn3 polypeptide can be wild-type.

In another embodiment, the yeast plasmid shuffling system allows the identification of agonists and antagonists that specifically affect expression of one Rrn3 polypeptide, but not another Rrn3 polypeptide. In a particular embodiment, a yeast strain that has a null allele of the endogenous yeast *RRN3* gene is rescued by an extrachromosomal copy of the yeast *RRN3* gene, or a *RRN3* nucleic acid from another eukaryotic organism. By screening essentially isogenic yeast strains, which differ only by the *RRN3* gene, agonists and antagonists can be identified which are specific for one eukaryotic *RRN3* nucleic acid or Rrn3 polypeptide, but not another *RRN3* nucleic acid or Rrn3 polypeptide. For example, yeast strains having a null allele of the endogenous yeast *RRN3* gene, and having either the human *RRN3* cDNA or the *Candida albicans RRN3* gene, could be screened for antifungal agents that specifically affect the fungal polypeptide. Similarly, agonists and antagonists can be identified that affect a particular allele or other derivative of a *RRN3* nucleic acid or Rrn3 polypeptide. For example, congenic mammalian cells, differing only in the *RRN3* gene or Rrn3 polypeptide, can be screened for candidate compounds that affect cell growth, viability and/or cell division.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (*see, e.g.*, Fields and Song, *Nature* 340:245-46 (1989); Chien *et al.*, *Proc. Natl. Acad. Sci. USA* 88:9578-82 (1991)) can be used to identify candidate compounds that specifically bind to a Rrn3 polypeptide or derivative.

Candidate compounds can also be identified by *in vitro* screens. For example, recombinant cells expressing *RRN3* nucleic acids can be used to recombinantly produce Rrn3 polypeptide for *in vitro* assays to identify candidate compounds that bind to Rrn3 polypeptide. Candidate compounds (such as putative binding partners of Rrn3 or small molecules) are contacted with the Rrn3 polypeptide (or fragment, derivative or analog thereof) under conditions conducive to binding, and then candidate compounds that specifically bind to the Rrn3 polypeptide are identified. Similar methods can be used to

screen for candidate compounds that bind to nucleic acids encoding *RRN3*, or a fragment, derivative or analog thereof. Methods that can be used to carry out the foregoing are commonly known in the art, and include diversity libraries, such as random or combinatorial peptide or non-peptide libraries that can be screened for candidate compounds that specifically bind to Rrn3 polypeptide. Many libraries are known in the art, such as, for example, chemically synthesized libraries, recombinant phage display libraries, and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor *et al.* (*Science* 251:767-73 (1991)), Houghten *et al.* (*Nature* 354:84-86 (1991)), Lam *et al.* (*Nature* 354:82-84 (1991)), Medynski (*Bio/Technology* 12:709-10 (1994)), Gallop *et al.* (*J. Med. Chem.* 37:1233-51 (1994)), Ohlmeyer *et al.* (*Proc. Natl. Acad. Sci. USA* 90:10922-26 (1993)), Erb *et al.* (*Proc. Natl. Acad. Sci. USA* 91:11422-26 (1994)), Houghten *et al.* (*Biotechniques* 13:412-21 (1992)), Jayawickreme *et al.* (*Proc. Natl. Acad. Sci. USA* 91:1614-18 (1994)), Salmon *et al.* (*Proc. Natl. Acad. Sci. USA* 90:11708-12 (1993)), International Patent Publication WO 93/20242, and Brenner and Lerner (*Proc. Natl. Acad. Sci. USA* 89:5381-83 (1992)).

Examples of phage display libraries are described in Scott and Smith (*Science* 249:386-90 (1990)), Devlin *et al.* (*Science* 249:404-06 (1990)), Christian *et al.* (*J. Mol. Biol.* 227:711-18 (1992)), Lenstra (*J. Immunol. Meth.* 152:149-57 (1992)), Kay *et al.* (*Gene* 128:59-65 (1993)), and International Patent Publication WO 94/18318.

In vitro translation-based libraries include, but are not limited to, those described in International Patent Publication WO 91/05058, and Mattheakis *et al.* (*Proc. Natl. Acad. Sci. USA* 91:9022-26 (1994)). By way of examples of nonpeptide libraries, a benzodiazepine library (*see, e.g.*, Bunin *et al.*, *Proc. Natl. Acad. Sci. USA* 91:4708-12 (1994)) can be adapted for use. Peptide libraries (*see, e.g.,* Simon *et al.*, *Proc. Natl. Acad. Sci. USA* 89:9367-71(1992)) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh *et al.* (*Proc. Natl. Acad. Sci. USA* 91:11138-42 (1994)).

Screening of the libraries can be accomplished by any of a variety of commonly known methods. See, for example, the following references, which disclose screening of peptide libraries: Parmley and Smith (*Adv. Exp. Med. Biol.* 251:215-18

(1989)); Scott and Smith (*supra*); Fowlkes *et al.* (*BioTechniques* 13:422-28 (1992)); Oldenburg *et al.* (*Proc. Natl. Acad. Sci. USA* 89:5393-97 (1992)); Yu *et al.* (*Cell* 76:933-45 (1994)); Staudt *et al.* (*Science* 241:577-80 (1988)); Bock *et al.* (*Nature* 355:564-66 (1992)); Tuerk *et al.* (*Proc. Natl. Acad. Sci. USA* 89:6988-92 (1992)); Ellington *et al.* (*Nature* 355:850-52 (1992)); U.S. Patent Nos. 5,096,815, 5,223,409, and 5,198,346, all to Ladner *et al.*; Rebar and Pabo (*Science* 263:671-73 (1994)); and International Patent Publication WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a Rrn3 polypeptide (or nucleic acid or derivative) immobilized on a solid phase and harvesting those library members that bind to the polypeptide (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith (*Gene* 73:305-18 (1988)); Fowlkes *et al.* (*supra*); International Patent Publication WO 94/18318; and in references cited hereinabove.

Animal Models:

The invention also provides animal models. In one embodiment, animal models for diseases involving cell hypoproliferation are provided. Such an animal can be initially produced by promoting homologous recombination between a *RRN3* gene in its chromosome and an exogenous *RRN3* nucleic acid that has been rendered biologically inactive (typically by insertion of a heterologous sequence, such as an antibiotic resistance gene). In one aspect, homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing the insertionally inactivated *RRN3* gene, such that homologous recombination occurs, followed by injecting the ES cells into a blastocyst, and implanting the blastocyst into a foster mother, followed by the birth of the chimeric animal ("knockout animal") in which a *RRN3* gene has been inactivated (*see* Capecchi, *Science* 244:1288-92 (1989)). The chimeric animal can be bred to produce additional knockout animals. Such animals can be mice, rats, hamsters, sheep, pigs, cattle, and the like, and are typically non-human mammals. In a specific embodiment, a knockout mouse is produced. Knockout animals are expected to develop, or be predisposed to developing diseases, involving cell hypoproliferation and can be useful to

screen for or test candidate compounds for the ability to promote proliferation and thus treat or prevent such diseases.

In a different embodiment of the invention, transgenic animals that have incorporated and express a functional *RRN3* gene have use as animal models of diseases involving cell hyperproliferation or malignancy. Transgenic animals are expected to develop or be predisposed to developing diseases involving cell hyperproliferation (*e.g.*, malignancy) and thus can have use as animal models of such diseases (*e.g.*, to screen for or test candidate compounds, such as potential anti-proliferation agents) for the ability to inhibit hyperproliferation (*e.g.*, tumor formation) and thus treat or prevent such diseases.

The following examples are provided merely as illustrative of various aspects of the invention and shall not be construed to limit the invention in any way.

Example 1

Cloning of human RRN3 cDNA

A BLAST search of the human Expressed Sequence Tag (EST) database using the yeast Rrn3 polypeptide sequence identified informative overlapping EST's (gb|AA481295; gb|AA191111; gb|N42382; gb|H43196; and gb|AA319333; which correspond to nucleotides 1233-854; 1129-1548; 1289-1546; 1621-1431; and 1554-1861 of SEQ ID NO:1) which encode a protein fragment with sequence similarity to amino acids 360 to 532 of the yeast Rrn3 transcription factor. Primers derived from the EST sequences were used to isolate a partial cDNA from human Jurkat cell RNA by reverse transcriptase-polymerase chain reaction ("RT-PCR"). Briefly, an oligonucleotide primer (hRACE 5'.2: 5'-TGATTGCAGCAAAAAAGTTAACCACTGA-3'; SEQ ID NO:3) complementary to the polynucleotide sequence encoding amino acids 508-517 of human Rrn3 was used to prime a first strand cDNA synthesis from 1 μg of total cell RNA (a gift of A.M. Hajjar, University of Washington) using Superscript reverse transcriptase (Gibco-BRL). Subsequent PCR with Pfu polymerase (Stratagene) using a gene-internal primer (hRACE 3'.1: 5'-CTATATCGCGACCTGATAAACATCTTTG-3'; SEQ ID NO:4), which corresponds to amino acids 343-352, and the hRACE 5'.2 primer (*supra*) produced PCR product which was inserted into the PCR vector pCR-BLUNT (Invitrogen) to

construct clone h3-2. DNA sequence analysis of this product confirmed that it was identical to the contiguous EST sequence.

The nucleotide sequence of this ORF-internal fragment (encoding amino acids 343 to 517 of the human polypeptide) was used to design primers to isolate further 5' and 3' sequence. The 5' end of the human mRNA was obtained by RT-PCR using a 5'RACE kit (Gibco). An oligonucleotide complementary to the 5' region of clone h3-2 (hRACE5'.3: 5'-CAAAGATGTTTATCAGGTCGCGATATAG; SEQ ID NO:5) was used to prime cDNA synthesis from human Jurkat cell RNA, and the cDNA product was purified, tailed and amplified by PCR according to the manufacturer's instructions. TdT-dependent PCR products derived from a single round of PCR amplification were cloned into the pCR-BLUNT vector to produce construct h5-L.

5'RACE generated two cDNA products which apparently arise from alternatively spliced mRNAs. Both cDNAs share identical 5' sequences including an initiator methionine preceded by an in-frame stop codon, indicating that they encode the N-terminus of the polypeptide and that they are transcribed from the same promoter. The longer product contains an additional 90 base pairs of sequence encoding amino acids 85 to 114 of the human polypeptide. Since this region shares sequence similarity with yeast Rrn3, the longer cDNA was used for further analysis.

The 3' region of the human RRN3 open reading frame was isolated by 3' RACE as follows: a poly A anchor oligonucleotide (Clontech) complementary to the 5' region of the poly A tail was used to prime a reverse transcription reaction using 1 μg of total RNA as described above. The resultant cDNAs were amplified by PCR using the gene-specific primer hRACE3'.1: 5' (SEQ ID NO:4) and the Clontech anchor primer. Subsequent analysis of the amplification products revealed that the 3' end of the open reading frame was missing. To determine the 3' end of the open reading frame, the partial human *RRN3* cDNA was used as a reference sequence to search the human dbEST databank. The longest EST identified by this search, gb|AA319333 (which corresponds to nucleotides 1554-1861 of SEQ ID NO:1), encodes an additional 70 amino acids which extend the sequence alignment of the human protein to within 8 amino acids of the yeast C-terminus, but does not appear to contain the stop codon identifying the end of the *RRN3* coding region.

To facilitate subsequent expression of the *RRN3* cDNA, a primer was designed which inserted a translational stop codon adjacent to the final conserved amino acid (glutamate 587). Briefly, the PCR products were secondarily amplified by nested PCR using primers hRACE3'.2: (5'-GGAAGCTTTTTGGCAAGAGCTAAA TTTATTCCTC-3'; SEQ ID NO:6) (encoding amino acids 410-419) and the EST AA319333 primer (5' GCGGATCCTCATTCAGCACT CATGTCTTCCCATACCTGATA-3': stop codon underlined; SEQ ID NO:7) to produce a ~500 base pair PCR product which was cloned into the pCR-BLUNT vector. Three independent clones of all PCR products described above were sequenced to avoid possible PCR generated errors. The EST AA319333 primer was used in conjunction with the ORF-internal 5' primer hRACE 3'.1 to amplify the sequence encoding the conserved C-terminal region of the human protein.

The resultant cDNA fragments were joined at overlapping restriction enzyme sites to produce a polynucleotide encoding a 587 amino acid polypeptide. Briefly, the human *RRN3* open reading frame was constructed by joining the three partial cDNA clones. The h5-L construct was cut at SacI site within the pCR-BLUNT vector, a blunt end created with Klenow enzyme, and the 3' end cleaved at an internal NruI site to generate a fragment which was inserted at the EcoRV and NruI sites of clone h3-2. The PCR product encoding the C-terminal region was then ligated at an internal HindIII site to generate the human *RRN3* construct used for further study. The integrity of the human *RRN3* ORF was confirmed by DNA sequence analysis.

Example 2

A recently reported human EST (gb|AW239267, which corresponds to nucleotides 1661 to 2068 of SEQ ID NO:1) encodes an additional 3' portion of the human *RRN3* cDNA. The full length cDNA thus encodes a polypeptide of 651 animo acids with a predicted molecular mass of 74 kD, which is similar to that of TIF-IA. The sequence of the human *RRN3* open reading frame has been deposited in the GenBank database (Accession No. AF227156).

Example 3

Genetic Locus for *RRN3*

The *RRN3* gene most likely has a single genetic locus. RT-PCR using the 5' and 3' primers adjacent the 5' and 3' ends of the open reading frame generates a product of the expected size and sequence. The cDNA sequence is contained within a genomic fragment of human chromosome region 16p12 spanning approximately 26 kb which is found on two independent bacterial artificial chromosomes ("BAC") clones (gb|AF001549 and gb|AC017077). All of the human EST sequences that were identified were correlated with these BAC clones.

Example 4

Preparation of Anti-Yeast Rrn3 Polypeptide Antibodies

The C-terminal region of yeast *RRN3* gene (residues 352-627) was subcloned into the pRSETC expression vector (Invitrogen) to generate a 6His-tagged fusion protein. This construct was transformed into *E. coli* strain UBS520, cultured in TBG/M9 medium containing both ampicillin and kanamycin (0.1mg/ml) to A600 =0.8, and protein expression was induced by addition of IPTG. The ~30 kD recombinant protein was isolated 3 hours post induction using Talon affinity resin (Clontech) under denaturing conditions according to the manufacturer's instructions. The protein was further purified by SDS-PAGE.

The excised gel slices were used for immunization of rabbits (R&R Rabbitry), according to standard methods. Antisera were fractionated by addition of solid ammonium sulfate to 50% saturation, the precipitate collected by centrifugation at 18k for 20 minutes in an SS-34 rotor, resuspended in 2 pellet volumes of 1x TBS, and dialyzed against 1x TBS overnight at 4° C.

Antiserum was used at a 1/2000 dilution in Western blots performed as described in Lin *et al.* (*Mol. Cell. Biol.* 16:6436-43 (1996)). For Western blot analysis, the bacterial cells were grown and expression was induced as described above except that 50 ml of medium was cultured, and lysates were prepared by boiling the harvested cells in SDS-PAGE buffer. Proteins were visualized using anti-His monoclonal antibodies (Qiagen) according the manufacturer's instructions.

Example 5

Immune Cross-Reactivity of Human and Yeast Rrn3 Polypeptide

To confirm that the human cDNA encodes a polypeptide which is related to yeast Rrn3, the human and yeast *RRN3* cDNAs were expressed in *E. coli* as 6-His fusion proteins and subjected to Western blot analysis. Full length yeast or human *RRN3* coding sequences were subcloned into pRSET vector (Invitrogen) to generate 6-His togged proteins for expression in *E. coli*.

Crude lysates prepared from bacteria expressing either the yeast gene or human cDNA or from cells transformed with the empty expression vector (pREST) were resolved by SDS-PAGE and then transferred to a hybridization membrane. The membrane was probed with anti-His monoclonal antibodies (Qiagen) or with antiserum generated against the C-terminal half of recombinant yeast Rrn3 (described in Example 4). The signal intensities obtained using antibodies against the 6-His tag were used to normalize the amount of recombinant protein loaded. Anti-His antibodies recognized polypeptides of 72 kD and 68 kD in lysates from cells expressing the yeast and human cDNAs, respectively, while no signal is observed in the lysate from the empty vector control. When these lysates were probed with antibodies generated against yeast Rrn3, the human polypeptide was more efficiently detected than the yeast polypeptide. This difference in recognition may be due to limited proteolysis of yeast Rrn3 C-terminus, since it is less stably expressed in bacteria than the human polypeptide. No signal is observed in the empty vector control lysate, however, indicating that the human polypeptide is specifically recognized by the anti-yeast Rrn3 serum, and it is clear from the strength of the observed cross-reaction signal that the yeast and human polypeptides are immunologically conserved.

Example 6

Construction of a Yeast *RRN3* Null Allele

To determine whether the human Rrn3 polypeptide could function in yeast, a yeast strain was constructed in which the yeast *RRN3* gene was disrupted.

A diploid yeast strain containing a disrupted *RRN3* gene was constructed as follows: The coding region of yeast *RRN3* was amplified from wild-type yeast genomic DNA and inserted into the pBluT vector (Novagen) to generate RRN3-BluT. RRN3-BluT was digested with EcoRV and PmeI, which cut in the *RRN3* coding region, and blunt ends were created using the Klenow fragment. A blunt-ended XhoI/BamHI fragment of the yeast *HIS3* gene was inserted into those blunt ends, creating a construct in which base pairs 492 to 1407 of the yeast *RRN3* open reading frame were replaced by the yeast *HIS3* gene to form an *rrn3Δ*::*HIS3* construct. The *HIS3* gene would then act as a marker for the presence of this gene disruption in yeast.

The *rrn3Δ*::HIS3 construct in RRN3-BluT was digested with SpeI to liberate a linear fragment which was then used to transform diploid wild-type strain W1665a/α (MAT a/α ade2-1 his3-11 leu2-3,112 trp1-1,15 ura3-1 can1-100 RAD5) to His^+, creating strain RLY300 (MAT a/α ade2-1 his3-11 leu2-3,112 trp1-1,15 ura3-1 can1-100 RAD5 rrn3Δ::HIS3). Sporulation of this yeast strain and dissection of the resulting spores (tetrads) yielded two viable and two inviable spores. The *HIS3* marker was not recovered from viable colonies. This is the expected segregation ratio if the rrn3Δ::HIS3 gene disruption has integrated at the *rrn3* locus and has inactivated ("knocked out") the *RRN3* gene on one chromosome.

The yeast *RRN3* gene was inserted into a yeast expression as follows to create plasmid pRRN3G-425. The coding region of *RRN3* was amplified from wild-type yeast genomic DNA and inserted into the pBluT vector (Novagen) to generate RRN3-BluT (described above). 476 base pairs of 5' yeast sequence was PCR-amplified and ligated to the *RRN3* open ready frame to produce the construct RRN3G. To express yeast *RRN3* from its own promoter, the RRN3G insert was subcloned into the PstI and BamHI sites of pRS425 to create plasmid pRRN3G-425, or into the SpeI and BamHI sites of pRS316 to create plasmid pRRN3G-316. Plasmid pRRN3G-425 carries the *LEU2* wildtype gene as a selectable marker and confers the Leu^+ phenotype on Leu^- auxotrophic strains.

Yeast strain RLY300 was transformed with pRRN3G-425 to Leu^+. Following sporulation of the transformed strain and dissection of the resulting spores, the spores were allowed to germinate on YPD plates. His^+ Leu^+ colonies were isolated and

designated RLY301 (MATa ade2-1 his3-11 leu2-3,112 trp1-1,15 ura3-1 can1-100 RAD5 *rrn3Δ*::HIS3 [pRRN3G-425]). Proper insertion of the rrn3Δ::HIS3 knockout at the *rrn3* locus in the His^+ progeny was confirmed by both PCR and Southern blot analysis. When grown under non-selective condition, all His^+ colonies retained the *LEU2* marker of pRRN3G-425, indicating that the episomal yeast *RRN3* gene could substitute for the inactivated yeast *RRN3* gene.

To obtain the yeast *RRN3* gene on a counterselectable plasmid, the yeast *RRN3* gene was subcloned into the *URA3* vector RLY302 to create plasmid RRN3G-316. The *URA3* gene is counterselectable using 5-fluoroorotic acid. The RRN3G-425 plasmid was exchanged for the RRN3G-316 plasmid using standard genetic techniques. Briefly, the RRN3G-316 plasmid was transformed into the RLY300 strain containing the RRN3G-425 plasmid. Following selection for cells transformed with the RRN3G-316 plasmid, the cells were grown under partially selective conditions so that the RRN3G-425 plasmid was lost. The resulting cells, that contained only the RRN3G-316 plasmid, were grown on 5-fluoroorotic acid (5-FOA) plates to select against the presence of the RRN3G-316 plasmid. No colonies grew on these plates, which demonstrated that the RRN3G-316 plasmid, containing the yeast *RRN3* gene, could rescue a yeast strain missing the endogenous yeast Rrn3 protein.

Example 7

Expression of Human *RRN3* in Yeast

The human and the yeast *RRN3* polypeptides were expressed as polyoma epitope-tagged fusion proteins from the yeast PGK promoter in $rrn3^-$ strain RLY303 (MATa ade2-1 his3-11 leu2-3,112 trp1-1,15 ura3-1 can1-100 RAD5 rrn3Δ::HIS3 [pNOY-TRP1]). Strain RLY303, a Nomura plasmid-dependent $rrn3^-$ strain, was created by transforming strain RLY302 (MATa ade2-1 his3-11 leu2-3,112 trp1-1,15 ura3-1 can1-100 RAD5 rrn3Δ::HIS3 [pRRN3G-316]) with pNOY-W (a derivative of pNOY103 (*see* Nogi *et al.*, *Proc. Natl. Acad. Sci. USA* 88:7026-30 (1991))). pNOY-W expresses the yeast 35S rRNA precursor under the control of the yeast GAL7 promoter. For this experiment, the *URA3* gene was disrupted by insertion of the *TRP1* gene.

Constructs for expression of polyoma-tagged Rrn3 polypeptides in yeast were generated as follows: a double-stranded oligonucleotide encoding two copies of the polyoma epitope and a single copy of the FLAG tag was inserted into a pRS425 derivative containing the yeast PGK promoter (Lin *et al.supra*). The coding regions of the yeast *RRN3* or human *RRN3* cDNA were PCR-amplified and inserted in-frame with the epitope cassette to create yPyWT and PyhRRN3, respectively. All constructs were confirmed by sequencing.

Strain RLY303 is only viable on galactose-containing media because the *RRN3* gene is only expressed from the PGK promoter on that media. RLY303 was transformed with either yeast wild-type *RRN3* (yPyWT), the human cDNA (PyhRRN3), or the control expression vector and grown under appropriate selection on galactose plates. The resulting transformants (Trp^+) were then streaked on CM-glucose plates to monitor *RRN3* function, or on CM-galactose plates as a positive control, and were incubated at 25° C, 30° C, or 37° C. When colonies were streaked onto glucose medium to repress transcription from the yeast GAL7 promoter, cells expressing either yeast or human Rrn3 grew at all temperatures tested, while those transformed with the empty expression construct were inviable.

The ability of the human cDNA to complement the $rrn3^-$ mutation demonstrates that its function in pol I transcription is conserved between yeast and humans and that the region of the human polypeptide encoded by the partial human *RRN3* cDNA is sufficient for *in vivo* activity in yeast. Interestingly, the yeast and human *RRN3* genes display distinct conditional phenotypes in the $rrn3^-$ background. Cells carrying the yeast gene grow well at 25° C and 30° C, but show slightly reduced growth at 37° C. In contrast, cells transformed with human *RRN3* cDNA grow better than yeast *RRN3* transformants at 37° C and show a pronounced reduction of growth at 25° C. These temperature-dependent growth differences are not observed when the transformed cells are maintained on galactose.

Expression of the human *RRN3* cDNA in yeast was verified by Western blot analysis. Briefly, yeast transformants RLY303 containing the yeast wild-type *RRN3* (yPyWT), the human *RRN3* cDNA (PyhRRN3), or the control expression vector were grown to O.D. 600=2 in 50 ml of galactose medium lacking leucine to select for the polyoma fusion expression constructs. Cells were harvested by centrifugation, washed

once with water, once with Yeast Extraction Buffer (YEB: 100 mM HEPES pH 7.9, 20% glycerol, 0.5 M KCl, 5 mM EGTA, 1 mM EDTA) and resuspended in 1.5 pellet volumes of YEB containing 2.5 mM DTT and protease inhibitors, as described by Lin *et al. (supra)*. An equal volume of glass beads was added, and cells were lysed by shaking 4 x 40 seconds in a FASTPREP® apparatus (Savant). The supernatant was transferred and spun in a microfuge for 20 minutes at 4° C. Fusion proteins were visualized by Western blot analysis using purified anti-polyoma epitope antibodies (Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952-54 (1985)) at a 1/5,000 dilution. The resulting Western blots demonstrated that the human *RRN3* cDNA was expressed in RLY303, as evidenced by anti-polyoma epitope binding to a polypeptide of the predicted molecular weight.

Example 8

Mutagenesis of the Yeast and Human *RRN3* Genes

To determine if the observed sequence conservation between yeast and human Rrn3 contributes to its function, the effect of an amino acid substitution at leucine 136 on the *in vivo* activity of the two polypeptides was compared. A temperature-sensitive mutant strain, which has a leucine to proline substitution at position 143 of yeast Rrn3, was used. This strain exhibits reduced growth at 37° C. Because the polypeptide sequence alignment indicates that this residue was conserved in the human Rrn3 polypeptide, a leucine to proline substitution was constructed at the corresponding amino acid position ("L136P") in human *RRN3* cDNA by site directed mutagenesis, as follows: PyhL136P was generated by site-directed mutagenesis (Kunkel, *Proc. Natl. Acad. Sci. USA* 82:488-92 (1985)) using oligonucleotide (5'-AACAGTCTGTGCTGATAC AGGATTACCAAGAAAAGCCAA-3'; SEQ ID NO: 8) (substitution underlined) to introduce a leucine to proline substitution at amino acid position 136 of human *RRN3* (PyhRRN3). The coding regions of the yeast *RRN3* gene, the yeast L143P mutant, the human *RRN3* cDNA and the human L136P *RRN3* mutant were amplified by PCR and inserted in-frame with the pRS425 epitope cassette to create vectors yPyWT, yPyL143P, Pyh*RRN3* and Pyh136P, respectively. All constructs were confirmed by DNA sequence analysis, and expression was verified by Western blot analysis.

The ability of the wild-type and mutant human polypeptides to complement the rrn3$^-$ knockout strain were examined. The temperature-sensitive phenotype of the

yeast L143P mutant is observed when cells are streaked to glucose plates or when serial dilutions of wild-type and mutant transformants are compared at 30° C and 37° C. In contrast, the human Rrn3 L136P mutant was unable to support growth on glucose at either temperature. To confirm that the Rrn3 L136P mutant was stably expressed, lysates were prepared from the transformants and were subjected to Western blot analysis using an antibody directed against the polyoma epitope, as described above. While full-length polypeptide is readily detected in all transformants, the levels of the human L136P mutant are reduced with respect to the human wildtype polypeptide. It is possible that increased expression of the human Rrn3 L136P mutant would result in a conditional phenotype similar to that observed with the yeast Rrn3 L143P mutant. It is clear, however, that the loss of *in vivo* function of Rrn3 L136P mutant is not solely the result of reduced expression because the human wildtype polypeptide is still capable of rescuing the rrn3$^-$ strain when it is expressed at levels comparable to those of hL136P. Therefore, the conserved leucine residue contributes to the *in vivo* function of both the yeast and human polypeptides.

Example 9

Identification of Other Eukaryotic RRN3 Genes

The yeast Rrn3 polypeptide sequence was used as a reference sequence in a BLAST search (Altschul *et al.*, *Nucl. Acids Res.* 25:3389-402 (1997)) to scan an EST database for other *RRN3* genes. Full length homologs were identified in *Schizosaccharomyces pombe* (sp|Q10110), *Caenorhabditis elegans* (sp|P48322), and three paralogs were identified in *Arabidopsis thaliana* (gb|AAD25746, gb|AAC16259, gb|AAC28984). Searching against unfinished genomic sequences reveals a near full length sequence from *Candida albicans* (gnl|Stanford_5476), and a partial sequence from *Drosophila melanogaster* mapping to chromosome 2 region 40D (gb|AC011757).

Rrn3 homologs in other eukaryotes are recognized by their similarity to these sequences. A partial list of Rrn3 EST's from other organisms includes mouse (gb|AA530643), zebra fish (gb|AI816698), *Botrytis cinerea* (emb|AL112233), rice (gb|AA752612), aspen (gb|AI164160), and poplar (gb|AI166768).

The previous examples are provided to illustrate but not to limit the scope of the claimed inventions. Other variants of the inventions will be readily apparent to those of ordinary skill in the art and encompassed by the appended claims. All publications, patents, patent applications and other references cited herein are hereby incorporated by reference.